STRUCTURE AND PHYSIOLOGY OF *PARIS*-TYPE ARBUSCULAR MYCORRHIZAS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

STRUCTURE AND PHYSIOLOGY OF *PARIS*-TYPE ARBUSCULAR MYCORRHIZAS

The overall aim of the work presented in this thesis was to increase our understanding of the morphology and physiology of *Paris*-type arbuscular mycorrhizas (AM).

Preliminary experiments were conducted to select a *Paris*-type AM suitable for glasshouse based experiments. A range of plant species were inoculated with AM fungi and their morphology assessed quantitatively. *Asphodelus fistulosus* when colonised by *Glomus coronatum* formed a *Paris-type* AM and was selected for use in the majority of the experiments in the thesis.

The time-course of development of the *A. fistulosus/G. coronatum* symbiosis was investigated. The mathematical relationships, or interdependence between the different structures was determined using a new method of colonisation assessment, the Interdependence Magnified Intersects Technique (IMIT). The time-course of development was slower than that of the *Arum-type* AM, supporting observations from field based studies. The IMIT revealed distinct interactions between the different structures.

The morphology and effect of *Paris-type* structures formed by *G. coronatum* when colonising *A. fistulosus* on the size and position of plant cortical cell nuclei were investigated using Laser Scanning Confocal Microscopy

(LSCM). Effects on the plant nuclei in cells containing arbusculate coils (AC) were similar to those observed in cells containing arbuscules of *Arum*-type AM. LSCM allowed for clear observation of these effects and of the morphology of *Paris-type* structures in general.

The growth and phosphorus (P) nutrition of *A. fistulosus* colonised by *G. coronatum* grown in soil with five different soil P concentrations was investigated, providing the first laboratory based assessment of the P nutrition of a *Paris*-type AM. The *A. fistulosus/G. coronatum* symbiosis was functional in terms of its effects on plant growth and P nutrition of the plant. There were significant effects of soil P concentration on the functioning of the symbiosis with important implications for the growth and survival of *A. fistulosus*.

In the final experiment, the importance of AM fungal identity in defining the morphology of AM was investigated using a range of Glomalean fungi colonising *Lycopersicon esculentum*. When colonising *L. esculentum* some AM fungi formed the *Arum*-type and others formed the *Paris*-type. The results challenge the traditional view that AM morphology is mainly under the control of the plant and provides a valuable opportunity unravel the significance of structural diversity in AM.

The research presented in this thesis has increased our knowledge of *Paris*-type AM and presents some new and exciting research opportunities.

PUBLICATIONS DURING CANDIDATURE

1. **Cavagnaro TR**, Smith SE, Smith FA, Ayling SM. (1999). The effect of P addition on growth of Leek (*Allium porrum* L.) and its fractional colonisation by *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders. *First Nationnal Conference on Mycorrhizas, Bogor, Indonesia, conference proceedings.*

2. **Cavagnaro TR**, Gao L-L, Smith FA, Smith SE. (2001). Morphology of arbuscular mycorrhizas is influenced by fungal identity. *New Phytologist.* **151(2):**469-476. (Bound at rear of thesis).

3. **Cavagnaro TR,** Kolesik P, Smith FA, Ayling SM, Smith SE. (2001). Arbuscular mycorrhizas formed by *Asphodelus fistulosus* and *Glomus coronatum*: Three-dimensional analysis of plant nuclear shift using laser scanning confocal microscopy**.** *Symbiosis* **30 (2-3): 109-122.** (Bound at rear of thesis).

4. **Cavagnaro TR**, Smith FA, Lorimer MF, Haskard KA, Ayling SM, Smith SE. (2001). Quantitative development of *Paris*-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum. New Phytologist* **149(1)** 105-113 (Bound at rear of thesis).

5. Bakhtiar Y, Miller D, **Cavagnaro TR**, Smith SE. (2001). Interactions between two arbuscular mycorrhizal fungi and fungivorous nematodes and control of the nematode with fenamifos. *Applied Soil Ecology* **17(2)**: 107-117.

6. Zhu Y-G, **Cavagnaro TR**, Smith SE, Dickson D. (2001). Backseat driving? Accessing phosphate beyond the rhizosphere-depletion zone. *Trends in Plant Science* **6(5)**: 194-195 .

DECLARATION

I declare that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University Library, being made available for loan or photocopying.

September 2001 *Signed*

Timothy R. Cavagnaro

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A straight line may be the shortest distance between two points, but it is by no means the most interesting.

Dr. Who

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" (I found it) but "That's funny..."

Isaac Asimov

This thesis is dedicated to the three people who have influenced me the most

Mum, Dad and Vanessa

CHAPTER 1 INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Arbuscular mycorrhizas (AM) are endomycorrhizas formed between fungi of the order Glomales and the roots of most terrestrial plant species (Morton & Benny, 1990). The order Glomales (Zygomycetes) contains six genera with approximately 150 described species (Morton & Benny, 1990). It has been estimated that 80% of angiosperms have the potential to form AM under their normal growth conditions (see Harley & Harley, 1987a, b; Newman & Reddell, 1987). These associations occur in most plant communities (Schwab, Menge & Tinker, 1991; Read, 1998) and are taxonomically the most widespread symbiotic association in nature.

Formation of AM usually results in the transfer of nutrients between the two partners (Smith & Read, 1997). The relationships between the plant and the fungi are complex. The establishment and quantitative aspects of AM differ between plant and fungal species and are influenced by a range of environmental conditions.

Despite the extensive occurrence of AM both geographically and within plant taxa, it has been possible to define two distinct morphological types, the

Arum-type and the *Paris*- type, described by Gallaud (1905). The two types are named after the plants in which they were first described, *Arum maculatum* and *Paris quadrifolia*. In an extensive survey of the literature (Smith & Smith, 1997), it was shown that the morphology of the AM associations in the angiosperms could be related to plant taxa at the family level. More plant families formed the *Paris*-type than the *Arum*-type, but some families contained plant species that formed both types or intermediate forms.

Although the *Paris*-type occurs in more plant families than the *Arum*-type, the majority of research has focused on the latter (Smith & Smith, 1997). In fact, all molecular research (Barker, Tagu & Delp, 1998; Harrison, 1999) and detailed time-course experiments of morphological development (Brundrett, Piché & Peterson, 1985; Rosewarne, Barker & Smith, 1997) have used the *Arum*-type. Although there are considerable structural differences between the two types, results from experiments using a limited range of *Arum*-type AM have often been considered as "representative" of both types. The research presented in this thesis is directed at improving our understanding of *Paris*-type AM. This review of the literature aims to provide the framework on which the research was conducted. Firstly, a general overview of the function and structural development of AM will be presented. Secondly, specific processes in AM relevant to this research will be reviewed.

1.2 The role of mycorrhizas

1.2.1 Benefits to the symbionts: an overview

The function of AM needs to be considered in terms of both the plant and the fungal partners. The plant can benefit from improved nutrition, drought resistance and resistance to pathogens, all potentially resulting in improved fitness and survival of the plant. The fungi benefit from a supply of organic carbon from the plant. With the exception of reserves stored in the spores of AM fungi (AMF), the only source of organic carbon for developing AMF is that supplied by the plant. Therefore, AMF are dependent on plants in order to grow to any significant extent, produce spores, and thus complete their life-cycle.

The most widely studied benefit to the plant is that of mineral nutrient acquisition, particularly phosphorus (P), by the fungi and subsequent transfer to the plant (Smith & Read, 1997). It has been demonstrated that up to 80% of plant P, 60% copper (Cu), 25% nitrogen (N), 25% zinc (Zn) and 10% potassium (K) can be delivered by the external hyphae of AMF (Marschner & Dell, 1994). These improvements in plant nutrition are of particular significance in soils of low nutrient status (Menge, 1983; Hetrick, 1991). However, most data are for herbaceous annuals, especially crop plant species which form *Arum*-type AM.

In addition to improved nutrition, plants which form AM can benefit from improved disease resistance (Dehne, 1982; Caron, Fortin & Richard, 1986; Perrin, 1990; St. Arnaud *et al.*, 1994). This occurs via the stimulation of disease resistance when the plants are contacted by the AMF, via the interaction of the AMF with the pathogen in the soil (St. Arnaud *et al.*, 1994) or within the root itself. Another reported benefit is that under drought stressed conditions, AMF can provide improved water uptake due to the hyphae being able to exploit a greater volume of soil and access smaller soil pores than roots can (Nelsen & Safir, 1982; Fitter, 1988; Osonubi, Bakare & Mulongoy, 1992; Koide, 1993). Hyphae of AMF can also indirectly benefit plants by improving aggregate stability and soil structure, through entanglement and enmeshment mechanisms and secretion of compounds which stabilise aggregates (Tisdall & Oades, 1979; Tisdall, 1991; Miller & Jastrow, 2000).

As a consequence of the benefits of forming AM, colonised plants have been found to have improved fitness, fecundity and seed quality/viability (Koide *et al.*, 1988; Shumway & Koide, 1995; Merryweather & Fitter, 1996). Ultimately, this wide range of benefits individually and combined can provide a competitive advantage to plants in the field.

1.3 Morphology and development of arbuscular mycorrhizas

AM have two principal components, the roots of plants and the hyphae of AMF. The hyphal component consists of both internal and external phases, which can in turn be further subdivided. The external phase includes spores, hyphae (both attached and unattached to the roots), vesicles (in some fungal species only), auxiliary cells (in some fungal species only) and appressoria on the surface of roots. The internal phase can include a range of different structures including arbuscules, hyphal coils, arbusculate coils, hypodermal entry coils (in some plant species only), intercellular hyphae, spores (in some fungal species only) and vesicles (in some fungal species only). The colonisation process and morphological development, focusing on the internal fungal morphology and features which distinguish *Arum*- and *Paris*-type AM will now be considered in detail.

1.3.1 Sources of inoculum

There are three types of AM propagules; spores, colonised root fragments, and active hyphal networks within the soil (Friese & Allen, 1991). The different propagules of AMF have evolved to satisfy different functions: dissemination, survival and infection of new plants. The spores contain lipids and thousands of

nuclei, and have thick walls. Spores are considered to be the main agents of dispersal of AMF and have been found in the soil at a wide range of densities. For example, Sutton & Barron (1972) found that spore numbers generally ranged from 20 to 70 spores. g^{-1} soil in soils with a range of crop species, whereas Gemma & Koske (1988) found densities ranging from 1 to 5 spores. g^{-1} soil, depending on the season when samples were taken. Although hyphae growing from spores can colonise roots, it is generally thought that colonised root pieces and hyphal networks in the soil are the main sources of inoculum under natural conditions (Read, 1987; Smith & Read, 1997). In fact, hyphal networks established in pots containing colonised plants have been successfully exploited as a means of experimental inoculation of plants with AMF (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997). Most research on AM propagules has been undertaken in a limited number of temperate environments. Thus, in environments such as deserts, where there are often long periods of time without plants to support a hyphal network, spores may in fact be very important in the colonisation of roots. There is no evidence to suggest that different types of propagules results in the formation of the different AM morphological types.

1.3.2 Pre-colonisation events

Hyphae of AMF growing in the soil, whatever their origin (spore, root fragment or active hyphal network), exhibit changes prior to physical contact with roots (Smith & Read, 1997). These changes are collectively termed the "pre-

colonisation events", and include structural modification of the hyphae such as extensive branching (Giovannetti *et al.*, 1993a, b) and as a consequence, a decrease in the diameter of hyphae. Giovannetti *et al.* (1993a, b) found that hyphae branched over the surface of host roots but not non-host roots. This demonstrates that prior to physical contact with a root, the hyphae of AMF are able to "recognise" a potential host from a non-host plant species. This recognition is related to the production of plant root exudates (Gianinazzi-Pearson, Branzanti & Gianinazzi, 1989; Giovannetti *et al.*, 1993a; Nagahashi, Douds & Abney, 1996). For example, exudates from roots of *Daucus carota*, containing quercetin and kaempferol, stimulate hyphal growth of *Gigaspora margarita* (Poulin *et al.*, 1993), whereas, the roots of some non-host species do not produce exudates which stimulate the growth of AMF (Gianinazzi-Pearson *et al.*, 1989; Giovannetti *et al.*, 1993a). It is thought that pre-colonisation events may increase the probability of contact between the plant and the fungi. Again there is no evidence to suggest that the contact and penetration of the root are related to the internal morphology of the resultant AM.

1.3.3 Contact and penetration of roots

Once the fungi have "identified" the roots of a potential host plant, appressoria are formed at the end of hyphae on the surface of roots. Appressoria have been observed on the surface of roots within 36 hours in ten day old seedlings of *Ocimum basilicum* and *Helianthus annus* after incubation with pre-germinated

sporocarps (Giovannetti & Citernesi, 1993). The branching and narrowing of hyphae and the formation of appresorium by hyphae when first contacting roots are all significant signs of recognition of a potential host by the fungus.

Following the formation of appressoria, the hyphae narrow to form a penetration peg followed by expansion after penetration. Mechanical pressure and the localised production of enzymes (Bonfante & Perotto, 1995) mediate this process. A number of enzymes have been identified as having a role in the penetration of plant cell walls by AMF, including cellulase, pectinase and xyloglucanase (Garcia-Garrido *et al.*, 2000). Bonfante & Perotto (1995) stated that very weak production of enzymes is localised to ensure that host viability is maintained, that defense responses are not triggered and that a high degree of compatibility is reached. Following this initial penetration, the hyphae may form coils within the hypodermis (where present) and the outer cortical cells, the importance of which are discussed below.

1.3.4 Internal phases of colonisation

In the *Arum*-type, the fungi form longitudinal intercellular hyphae in the air-spaces between the cortical cells. The intercellular hyphae then form short side branches which penetrate cortical cells to form arbuscules: usually defined as highly branched haustoria within plant cells which can arise from any part of the main intercellular hyphae (Gallaud, 1905; Gerdemann, 1968). In some *Arum*-type

AM, hyphal coils are formed within the passage cells of the hypodermis. However, these coils do not usually make up a large component of the intracellular fungal biomass (Smith & Read, 1997). A representative *Arum*-type infection unit is shown in Figure 1.1 a.

In the *Paris*-type the intercellular phase of colonisation is absent, or virtually so. Instead, the *Paris*-type is characterised by the formation of extensive intracellular hyphal coils and arbusculate coils in the root cortex, spreading directly from cell to cell (Gallaud, 1905). The arbusculate coils are similar to hyphal coils, except that they have small highly branched arbuscule-like structures on their surface (Gallaud, 1905; Yawney & Schultz, 1990). As with the *Arum*-type, entry coils can also be formed in the hypodermis of roots of *Paris*-type AM (Gallaud, 1905). A representative *Paris*-type infection unit is shown in Figure 1.1 b.

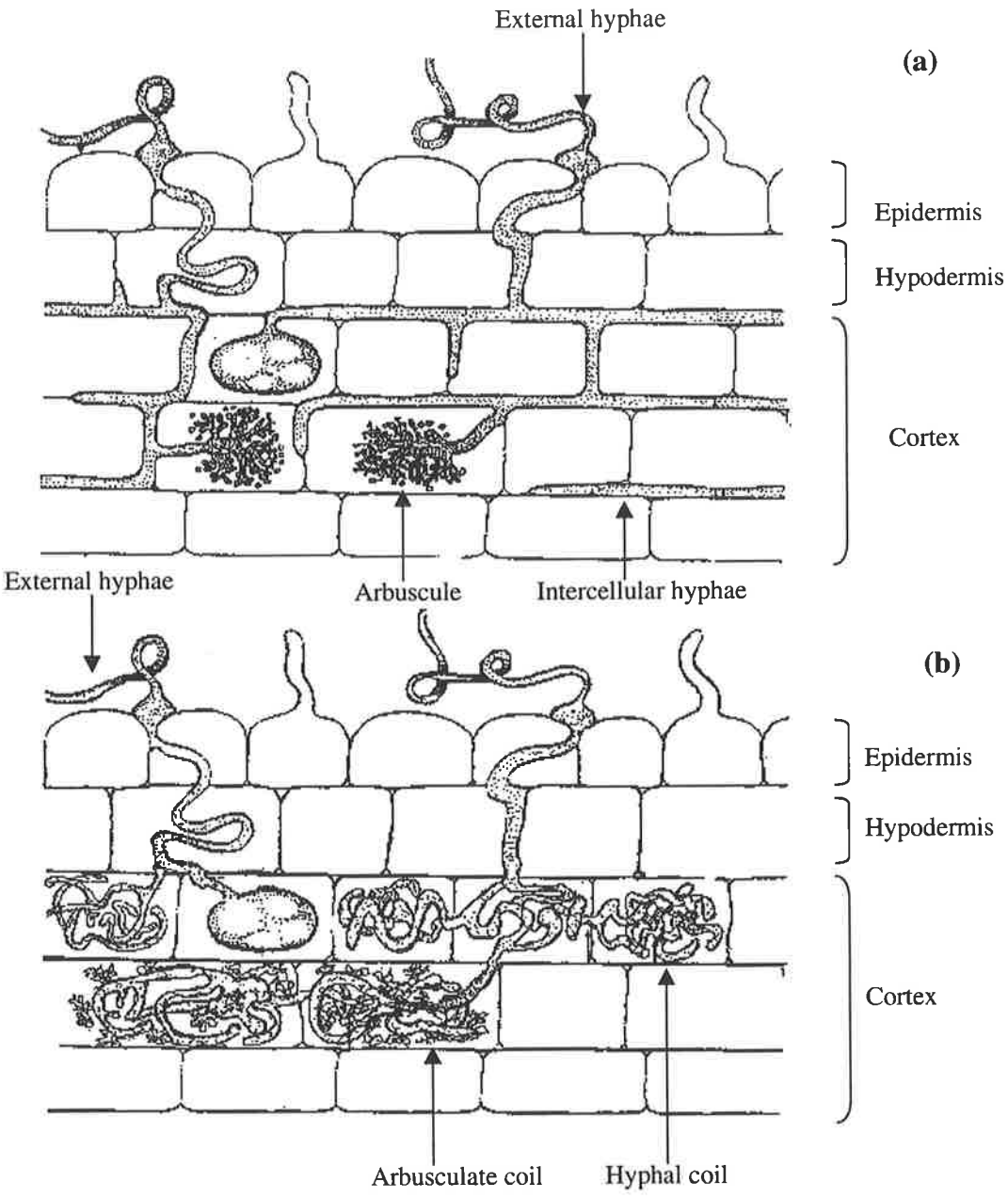


Figure 1.1 Schematic diagram of morphological features of (a) *Arum*-type and (b) *Paris*-type AM (modified from Dickson, 1999).

Of all the structures formed by AMF within roots, the arbuscule has received the most attention. When the side branches of intercellular hyphae enter cortical cells to form arbuscules, they do not penetrate the plasma membrane (periarbuscular membrane), but rather, they invaginate it (Cox & Tinker, 1976; Toth & Miller, 1984; Toth, Page & Castleberry, 1984; Smith & Gianinazzi-Pearson, 1988; Dexheimer & Pargney, 1991). As a result, the hyphae do not come in direct contact with the plant cells cytoplasm and they develop within an apoplastic compartment (Smith & Read, 1997). After initial penetration of the cortical cell, the trunk hyphae branch dichotomously to form the arbuscule.

The development of arbuscules is a rapid process, with the first arbuscules being observed within 2 to 5 days after initial hyphal penetration of the root (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997). Similarly, the turnover of arbuscules is relatively rapid. On average arbuscules of *Glomus mosseae* formed in *Allium cepa* live for four days (Cox & Tinker, 1976). The total arbuscular life-cycle, that is, the time between the formation of the first arbuscules to when the first degenerated arbuscules were noted, ranges from 4 to 12 days (Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988). Typically, the oldest arbuscules are found close to the initial point of entry of the fungus into the root. The location of arbuscules can differ between different plant and fungal species. For example, a pattern of arbuscule production in cells of the inner cortex around the stele of the root has been observed in *Trifolium subterraneum* (referred to as *T. subterraneum* hereafter) inoculated with a range of *Glomus* species (Abbott,

1982). Similarly, Blee & Anderson (1998) also observed that arbuscules were only formed immediately adjacent to the endodermis in *Zea mays*, *Triticum aestivum*, *Daucus carota*, *T. subterraneum*, *Vigna radiata* and *Phaseoleus vulgaris*. However, other plant species such as *Allium porrum* (referred to as *Al. porrum* hereafter) form arbuscules in a scattered arrangement throughout the layers of cortical cells.

The morphology and ultrastructural development of *Paris*-type AM have received a lot of attention, whereas the functioning of this type has not (for example, Gallaud, 1905; Kinden & Brown, 1975a, b, c; 1976; Bonfante-Fasolo & Fontana, 1985; Brundrett & Kendrick, 1990a, b; Yawney & Schultz, 1990; Cooke, Widden & O'Halloran, 1993; Whitbread, McGonigle & Peterson, 1996; Imhof, 1999). Yawney & Schultz (1990) studied the morphology of AM formed by *Acer saccharum* (referred to as *Ac. saccharum* hereafter) inoculated with *Glomus etunicatum*. The hyphae forming the coils ranged in size from 2 to 5 μm in diameter. The diameter of the arbuscular branches on the surface of the coils ranged from 0.4 to 0.8 μm and originated from more than one point on each coil simultaneously. *Panax quinquefolius* (referred to as *Pa. quinquefolius* hereafter) is another *Paris*-type plant which has been studied in some detail, largely from field collected material where the AMF identity was unknown (for example Whitbread *et al.*, 1996; Melville *et al.*, 1998). In this species, the fungi form hyphal coils which can develop into arbusculate coils. The hyphae forming the coils then grow from cell to cell with noticeable constrictions when passing

through cell walls, as observed with other *Paris*-type AM (Yawney & Schultz, 1990). Kinden & Brown (1975a, b, c; 1976), using electron microscopy, studied in detail the AM formed by *Liriodendron tulipifera* (referred to as *Li. tulipifera* here after) and indigenous soil fungi (tentatively identified from co-occurrence of spores as *G. mosseae*). They found that extensive hyphal coils were formed with the cell to cell growth, typical of the *Paris*-type. The hyphal coils were multi-nucleate, aseptate and contained all the organelles necessary for normal metabolic activity (for Glomalean fungi). Bonfante-Fasolo & Fontana (1985) investigated the AM formed by *Ginkgo biloba* and several Glomalean fungi, and concluded that the cellular organisation was "identical" to that of other AM described at that time (*Arum*-type AM). They also observed that the plasmamembrane of the cortical cell was invaginated, as occurs in cells of *Arum*-type AM containing arbuscules. Dickson & Kolesik (1999) calculated the volume of representative arbuscules and hyphal coils of *Arum*- and *Paris*-type AM and found that the two structures have similar volumes.

The location of hyphal coils and arbusculate coils in roots has been studied in the *Paris*-type. In *Li. tulipifera* (Kinden & Brown, 1975a) coils were typically formed in the outer cortex and arbuscules (Kinden & Brown, 1975a referred to arbusculate coils as arbuscules in their papers) in the inner cortex, whereas in *Pa. quinquefolius* hyphal coils and arbusculate coils were located randomly within the root cortex (Whitbread *et al.*, 1996). The morphology of *Paris*-type AM has been

described in detail. However, quantitative information is lacking about the structural development of this morphological type

In addition to the two main morphological types of AM, there are also a number of reports of AM with an intermediate morphology. The occurrence of AM with an intermediate morphology was reviewed by Smith & Smith (1997), and was found to occur in fewer plant families than *Arum*- and *Paris*-type AM. Plants classified as having intermediate type AM have two origins. Firstly, where there are examples of a single plant species of genus that has an *Arum*-type morphology in one report and a *Paris*-type morphology in another. For example Greny (1973) reported *Zea mays* as having an *Arum*-type morphology, whereas Johnston (1949) recorded it as having a *Paris*-type morphology (cited in Smith & Smith, 1997). Secondly, plants from roots of a single plant containing features of both morphological types, for example, hyphal coils and intercellular hyphae in the same root system, are also classified as intermediates. For example, the Apocynaceae have hyphal coils and intercellular hyphae (Weber, Klahr & Marronheimbuch, 1995). The distinction between these two reasons for the classification of intermediate type AM is important (Smith and Smith, 1997) and is considered in Chapter 8. The morphological features of the two types and intermediate types are summarised in Table 1.1.

In addition to intermediates there are AM with "irregular" structures. For example, intracellular hyphal swellings termed "bobbits" were described in

Clintonia sp.and *Medeola* sp. (Widden, 1996). Gallaud (1905) also noted similar structures in *Colchicum automnale*. The occurrence of these "bobbits" in other AM is not known and they have received little attention.

Table 1.1 Main morphological features of the *Arum*-type, *Paris*-type and intermediate morphological types. Note: ✓/✘ = presence/absence.

Structures	***Arum*-type**	***Paris*-type**	**Intermediate**[1]
External hyphae (EH)	✓	✓	✓
Intercellular hyphae (IH)	✓	✘	✓ or ✘
Arbuscules (A)	✓	✘	✓ or ✘
Hypodermal entry coils (EC)	✓ or ✘	✓ or ✘	✓ or ✘
Hyphal coils (HC)	✘	✓	✓ or ✘
Arbusculate coils (AC)	✘	✓	✓ or ✘
Vesicles[2] (V)	✓ or ✘	✓ or ✘	✓ or ✘

[1]Intermediate types have structures that are formed by both *Arum*- and *Paris*-type AM.

[2]The presence of vesicles will depend on the species of fungus colonising the plant.

1.4 Control of arbuscular mycorrhizal morphological types

Studies of different plant and fungal combinations have suggested that AM morphological type is largely dependent on the plant species (Smith & Smith, 1997). Smith & Smith (1997) concluded that 30 families formed the *Arum*-type, 41 families formed the *Paris*-type and 21 families formed either an intermediate morphology or had members with both the *Arum*- and *Paris*-types (see Table 1.2). The role of the plant in controlling the morphological type of AM was probably first demonstrated experimentally by Barrett (1958), who found that the same Glomalean isolate formed the *Arum*-type in *Zea mays* and the *Paris*-type in *Solanum tuberosum*. Similar results have been obtained in a few other studies with other plant and fungal species (for example, Gerdemann, 1965; Jacquelinet-Jeanmougin & Gianinazzi-Pearson, 1983). It has been suggested that AM morphology is controlled via the presence or absence of extensive air-spaces in roots (Brundrett & Kendrick, 1988; 1990b). That is, in the *Arum*-type the hyphae grow along intercellular air-spaces giving the intercellular hyphae, whereas in the absence of these air-spaces, the *Paris*-type is formed with its characteristic cell to cell growth. Further evidence suggesting that the plant is controlling morphology is that the *Paris*-type tends to be found in longer-lived roots than the *Arum*-type (Brundrett & Kendrick, 1990a, b).

Table 1.2 Family groupings of angiosperms having distinct *Arum*-type and *Paris*-type VA mycorrhizas and those with both types and/or intermediate types. Modified from Smith & Smith (1997).

	***Arum*-types**	***Paris*-types**	**Both types (B) and/or intermediate types (I)**
Monocots	Agavaceae (1) Araceae* (5) 'Liliaceae': *-Alliaceae* (1) *-Asphodelaceae* (1) *-Anthericaceae* (1) *-Convallariaceae* (4) *-Hyacinthaceae* (4) *-Hypoxidaceae* (1) *-Ruscaceae* (1) Zingiberaceae (1)	Burmanniaceae (1) Cannaceae (1) Dioscoraceae (2) Heliconiaceae (1) 'Liliaceae': *-Colchicaceae* (2) *-Liliaceae s. s.* (1) *-Trilliaceae* (3) *-Uvulariaceae* (2) Marantaceae (1) Thismiaceae (1) Triuridaceae (1)	Araceae* (1P)[7] Gramineae (>10) (B,I) Arecaceae (3) (B) Pandanaceae (1) (I)
Dicots	Alangiaceae (1) Anacardiaceae (1) Asclepiadaceae* (4) Balsaminaceae (1) Begoniaceae (1) Boraginaceae (2) Campanulaceae (1) Combretaceae (1) Compositae (5) Cucurbitaceae (2) Elaeocarpaceae (2) Guttiferae (2) Malvaceae (5) Oleaceae (1) Proteaceae (1) Rosaceae (2) Staphyleaceae (1) Symplocaceae(1) Thymeleaceae (1) Turneraceae (1) Urticaceae (2) Vitaceae (1)	Aceraceae (1) Annonaceae (1) Araliaceae (2) Aristolochiaceae (1) Bombacaceae (2) Caricaceae (1) Casuarinaceae (1) Cecropiaceae (1) Cornaceae (1) Cunoniaceae (1) Gentianaceae (7) Grossulariaceae (1) Hamamelidaceae (1) Hippocastanaceae (1) Lecythidaceae (1) Loganiaceae (1) Magnoliaceae (2) Malpighiaceae (1) Melastomaceae (1) Moraceae (2) Myrsinaceae (1) Myrtaceae (1) Polygalaceae (1) Rubiaceae* (6) Sapindaceae (1) Saxifragaceae (1) Theaceae (1) Ulmaceae (2) Violaceae (1)	Apocynaceae (14) (B,I) Asclepiadaceae* (I) Burseraceae (1) (I) Caprifoliaceae (1) (I) Euphorbiaceae (6) (B, I) Flacourtiaceae (1) (I) Labiatae (7) (B) Leguminosae: *-Caesalpinoideae* (1) (I) *-Mimosoideae* (3) (B) *-Papilionoideae* (13) (B) Linaceae (1) (B) Meliaceae (1) (I) Menyanthaceae (2) (I) Ranunculaceae (3) (B) Rubiaceae* (1A) Rutaceae (6) (B, I) Scrophulariaceae (2) (B) Solanaceae (4) (B) Sterculiaceae (2) (I) Umbelliferae (6) (I) Verbenaceae (5) (B)

(1, etc) = number of genera recorded, (B) = both types recorded, (I) = intermediate characters (extensive coils and IH). Araceae* (etc) in two columns occurs where there's a single record that differs from majority; (1P) or (1A) indicates minority record in 'Both' category.

An important point that was considered by Smith & Smith (1997) was the potential role of the fungal genome in influencing the morphology of AM. A few studies have revealed some difference in the morphology of AM in a single plant species when colonised by different fungal species. For example, Abbott (1982) developed a key to identify the fungal species colonising the roots of *T. subterraneum* based on the internal morphology of colonisation by ten different Glomalean fungi. This key is important as it has allowed for identification of species of AMF from both field soils and in experiments using inoculated plants (for example, Abbott & Robson, 1982; Wilson, 1984; Scheltema *et al.*, 1987). Abbott, Robson & Gazey (1992) also reported different patterns of hyphal development within and outside the roots depending of the fungal species. When a *Glomus* species penetrates a root the intracellular hyphae form an infection unit which extends along the length of the root. Where there are multiple entry points the infection units merge into each other. Alternatively, in *Scutellospora calospora* a series of shorter infection units are formed, which do not merge into one another if there are multiple points of root colonisation. Morphological differences between fungal species colonising roots are also described by Brundrett *et al.* (1996). Brundrett *et al.* (1996) showed that *S. calospora* formed thick hyphae which tended to form loops near the entry points compared to *Glomus* species which had thinner hyphae and did not form loops close to the entry points. Furthermore, the infection units formed by *S. calospora* were shorter than those of the *Glomus* species. Variation in the morphology of AM formed by *Daucus carota* have been observed (Söderström *et al.*, 1996) with

extensive coils and few arbuscules formed by *G. coronatum*, but the reverse by *G. mosseae*. There is clearly a need for further investigations of the factors controlling AM morphology.

1.5 Absence of arbuscules in arbuscular mycorrhizas

It is often assumed that arbuscules are the site of nutrient transfer (especially P) in AM. This is largely due to the fact that surface area of the interface between the arbuscule and the plant cell membrane is large. However, in the *Paris*-type, hyphal and arbusculate coils rather than arbuscules are formed. A number of papers cite situations where arbuscules and arbusculate coils are either present in low numbers or completely absent (Allen & Allen, 1984; Pocock & Duckett, 1984; Duckett & Ligrone, 1991; Koske, Gemma & Flynn, 1992; Muthukumar, Udaiyan & Manian, 1996; Muthukumar *et al.*, 1997). There are also reports of the density of arbuscules (Gay, Grubb & Hudson, 1982; Brundrett *et al.*, 1985; Whitbread *et al.*, 1996; Rosewarne *et al.*, 1997) and metabolic activity (Smith & Dickson, 1991) changing with time and P nutrition of the plant (Mullen & Schmidt, 1993). Often reports of AM with no arbusucles come from plants which have low levels of colonisation. For example, Muthukumar *et al.* (1996) reported that *Cyperus rotundus* (refereed to as *C. rotundus* hereafter), from field collected material did not have arbuscules, however, the percentage of the root length colonised was also low. *Salsola kali* was reported to not have arbuscule or vesicles, however, there was little internal colonisation otherwise (Allen & Allen,

1984). If a plant and fungal combination in which a developmental stage when few or no arbuscules are formed, but "normal" levels of total colonisation were present could be found, it may be useful in providing insight into whether or not arbuscules are the only site involved in nutrient transfer. This would involve finding either a *Paris*-type AM which did not form arbuscules or a developmental stage of an *Arum*- or *Paris*-type AM when no arbuscules were formed.

1.6 Time-course of development

Time-course studies under controlled conditions of the development of different structures in *Arum*-type AM have been undertaken. In time-course studies it is important to use synchronously colonised plants to ensure that associations of a similar age are used. This can be achieved using nurse-pots (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997). Nurse-pots are pots containing mycorrhizal plants, into which the plants of interest are transplanted, providing near-synchronously colonised root material (outlined in detail in Chapter 2). Using nurse-pots, the time-course of development of *Al. porrum* and *Glomus versiforme* (Brundrett *et al.*, 1985), *Lycopersicon esculentum* (referred to as *Ly. esculentum* hereafter) and *G. intraradices* (Rosewarne *et al.*, 1997), and *Hordeum vulgare* and *G. intraradices* (G. Delp, personal communication), have been investigated. The time-course of development of these associations was rapid. For example, in *Ly. esculentum* and *Al. porrum*, 25% and 15% of the root length had external hyphae respectively two days after transplanting (Brundrett *et al.*, 1985; Rosewarne *et al.*,

1997). In *Ly. esculentum*, the maximum external hyphal colonisation was reached within eight days after transplanting (Rosewarne *et al.*, 1997), whereas in *Al. porrum* a peak in colonisation by external hyphae was reached 6 days after transplanting. The first arbuscules were observed 2 to 5 days after initial hyphal penetration and reached maximum numbers at 8 and 16 days (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997) (note, the experiment using *Al. porrum* did not continue past 8 days after transplanting). These studies demonstrate that the formation of *Arum*-type AM is a rapid process, whereas it has been suggested that the time-course of *Paris*-type AM may be relatively slow (Brundrett & Kendrick, 1988). If this is true, it is probably due to the more tortuous path that hyphae follow and the greater number of cell walls that hyphae have to cross in the *Paris*-type compared to the *Arum*-type. However, this suggested slower time-course of the *Paris*-type is yet to be verified under controlled experimental conditions.

Almost all studies of AM require a measure of colonisation at some stage of the investigation. The extent and detail in which this colonisation is investigated depends on the aims of each particular study. As a consequence of the differing aims of individual studies, a number of methods for the quantification of root colonisation have been developed. These methods include visual measures (Ambler & Young, 1977; Giovannetti & Mosse, 1980; Trouvelot, Kough & Gianinazzi-Pearson, 1986; McGonigle *et al.*, 1990), chemical estimation of chitin (Hepper, 1977; Bethlenfalvay, Pacovsky & Brown, 1981), colorimetric methods (Hepper, 1977), image analysis (Smith & Dickson, 1991) and

quantitative molecular methods (Edwards, Fitter & Young, 1997; Antoniolli, 1999).

If the aim is to quantify the length of root colonised, then the most appropriate, and widely used method is the grid line intersect method (Giovannetti & Mosse, 1980). However, if the experiment requires information about the occurrence of different structures in, for example, time-course experiments, then the Magnified Intersects Technique (MIT) (McGonigle *et al.*, 1990) would be the most appropriate method. Briefly, the MIT uses high magnification to score the occurrence of different structures within the root which intersect a crosshair in the eyepiece of a microscope at random points along the root (outlined in detail in Chapter 2). The MIT has been used to assess the influence of plant and fungal identity on AM morphology (Muthukumar *et al.*, 1996; Rosewarne *et al.*, 1997; van der Heijden *et al.*, 1998a), as well as varied environmental conditions (Braunberger, Miller & Peterson, 1991; Pearson, Smith & Smith, 1991;

Muthukumar *et al.*, 1997; and many others). As a result, this method has had a large impact on the advancement of our knowledge of AM.

A frequently overlooked aspect of quantification of colonisation, is the way in which the data are analysed. Some useful methods of data analysis are often overlooked and potentially valuable information is lost. Furthermore, incorrect analysis of data can potentially give misleading results. A few authors have discussed the importance of methods of data analysis with respect to

colonisation in studies of AM (St John & Hunt, 1983; McGonigle, 2001). However, this is an important area which has received little attention.

Although many methods for assessment of colonisation and subsequent data analysis have been described, there is currently no method to determine the quantitative relationships between the different structures, and how they change relative to each other over time. This information is useful if we want to increase our understanding of the relationships between the different structures. For example, in studies of *Arum*- and *Paris*-type AM, these relationships may differ and it would be useful to be able to demonstrate this quantitatively, rather than only qualitatively.

1.7 Cellular development and Laser Scanning Confocal Microscopy

In addition to the plant/fungal structural interactions described above, there are other effects on the plant at a much smaller scale. For example, effects of colonisation on the nucleus of colonised cells have been demonstrated. It has been established that in cells containing arbuscules, there is an increase in the size of the plant nucleus (hypertrophy) (*Al. porrum/Glomus* sp. -*Arum*-type) (Blair, Peterson & Bowley, 1988; Berta *et al.*, 1990) it becomes lobed, the chromatin is decondensed (*Al. porrum/Glomus* sp.- *Arum*-type) (Balestrini, Berta & Bonfante,

1992), and there is a delay in nuclear senescence (*Al. porrum/G. mosseae- Arum*-type) (Lingua *et al.*, 1999). There is also a positional change of the plant nuclei from the periphery towards the centre of cells containing arbuscules (*Al. porrum/Glomus* spp.- *Arum*-type) (Balestrini *et al.*, 1992). Balestrini *et al.* (1992) observed no change in the position of the nuclei of *Al. porrum* in hypodermal cells containing entry coils. However, there have been no quantitative investigations of the influence of *Paris*-type structures on the size and position of plant nuclei, only qualitative observations (for example, Gallaud, 1905; Kinden & Brown, 1975c).

Nuclei of AMF have also been studied. Bago *et al.* (1998b, 1999) found that the nuclei were more abundant in thin runner hyphae and branched absorbing structures (BAS) compared to thick runner hyphae of AMF. They also found that the nuclei were spaced evenly along the length of the hyphae. Timonen, Smith & Smith (2001) found that there were many nuclei within the intercellular hyphae and arbuscules of *Arum*-type AM. The nuclei of AM fungi when forming *Paris*-type structures have been studied in very little detail.

1.8 Phosphorus and arbuscular mycorrhizas

1.8.1 Effects of phosphorus on plant growth: mechanisms of uptake, translocation and transfer

The most widely studied benefits of forming AM are in terms of mineral nutrition, mainly P. Improvements in plant P nutrition due to the formation of AM have been well documented. Sanders & Tinker (1971) demonstrated that there was increased P uptake per unit root length in colonised plants compared to uncolonised plants. It has since been shown that the influx of P in colonised roots can be 3 to 5 times higher than in non-colonised roots (Smith & Read, 1997). Again, these studies have used *Arum*-type AM and the *Paris*-type has been largely ignored. The mechanisms of P uptake, translocation and transfer to the plant have received much experimental attention and are outlined below.

Phosphorus is usually taken up by the plant in the inorganic form, $H_2PO_4^-$ (Schachtman, Reid & Ayling, 1998). As plants take up nutrients from the soil, a zone of nutrient depletion can form around the roots. This zone of depletion is particularly relevant with respect to relatively immobile nutrients such as P and Zn. The principal mechanism of AM-mediated nutrient acquisition is the growth of fungal hyphae to a region beyond the depletion zone. The hyphae actively take up nutrients and transfer them to the plant from sources which would not have been accessible to the plant otherwise. This was demonstrated in experiments

using ^{32}P tracers (Rhodes & Gerdemann, 1975; Jakobsen, Abbott & Robson, 1992a, b; Johansen, Jakobsen & Jensen, 1993; and others). Such experiments have shown that P can be taken up from more than 11 cm from the surface of the host roots (Li, George & Marschner, 1991), a distance much greater than plants are able to take up P from.

Movement of P in AM systems occurs in several steps: uptake by the external hyphae, translocation along the external hyphae to fungal structures within the plant root, transfer of P into the interfacial apoplast between the plant and fungi and finally uptake of P by the plant. Given that the P concentration in the soil solution is often <10 μM and the concentration of P in the hyphae is several orders of magnitude higher, uptake of P from the soil by hyphae is an active process (Ayling, Smith & Smith, 2000). Consistent with this, a high affinity fungal P transporter (GvPT) has been cloned from the external hyphae of *Glomus versiforme* (Harrison & van Buuren, 1995). GvPT is only expressed in the external hyphae of AM and not in the internal phase of colonisation. The external hyphae of AMF show modifications which are thought to play a role in the uptake of P from the soil. For example, Bago *et al.* (1998a) reported the occurrence of branched absorbing structures (BAS), which develop after a symbiosis has been formed by a fungal germ tube and a root in axenic culture. These BAS may be important in acquisition of nutrients.

As with many other microbial systems, once P is taken up by the fungi it is stored in vacuoles (in the external hyphae), where it is converted into polyphosphate, an energy-rich form of phosphate. Phosphate is stored in this form as polyphosphate has smaller effects of the osmotic potential of cells than orthophosphate, thus preventing large-scale efflux of phosphate. Solaiman *et al.* (1999) demonstrated that in *Gigaspora margarita*, polyphosphate constituted 5 to 17% of the total P in the hyphae. In the ectomycorrhizal fungus *Pisolithus tinctorius* the polyphosphate is stored in a soluble form in association with K^+ (Orlovich & Ashford, 1993). It was previously thought that polyphosphate was stored in granules (Cox & Tinker, 1976), however, the granules are an artifact of sample preparation (Orlovich & Ashford, 1993). Polyphosphate has been implicated as having a role in P translocation in AM by a number of workers (Cox & Tinker, 1976; Solaiman *et al.*, 1999). Although the transport mechanism of P within the hyphae to the plant has not been determined experimentally, it is thought that polyphosphate is transported via a vacuolar transport system as in ectomycorrhizas (Ashford, 1998). This needs further investigation.

Once P is transported along the hyphae it is transferred to the plant. Woolhouse (1975) suggested that transfer of P from the fungus to the plant occurred as a two step process. Firstly, P is passively effluxed into the apopolastic space between the plant and fungal plasma-membranes, and secondly, taken up actively by the plant, for example, via proton-coupled co-transport. In support of this theory, a number of studies have shown a high ATPase activity on

the peri-arbuscular membrane histochemically (Marx *et al.*, 1982; Gianinazzi-Pearson *et al.*, 1991), in arbuscule-containing cells of *Ly. esculentum* using *in situ* hybridisation (Rosewarne *et al.*, 1999), and immunologically in *Nicotiana* sp. (Gianinazzi-Pearson *et al.*, 2000). These studies all implicate arbuscules as a site of P transfer from the fungus to the plant. An important point that is often overlooked is the importance of interfaces between the plant and the fungi aside from the arbuscule. This point was addressed by Gianinazzi *et al.* (1991) who investigated the distribution of ATPases adjacent to the intercellular hyphae as well as on the peri-arbuscular membrane. Briefly, they concluded that there was some activity at the plant cell/intercellular hyphae interface which may be related to uptake of carbon by the fungi, suggesting spatial separation of nutrient uptake processes in AM. However, the interfaces between the plant and hyphal coils and arbusculate coils have not been investigated directly, although the need for much work was emphasised by (Smith and Smith, 1996). Furthermore, in other mycorrhizal systems, such as orchid mycorrhizas, there is no arbuscular phase of colonisation. Therefore, other interfaces must be involved in transfer of nutrients between the symbionts.

1.8.2 Effects of phosphorus on colonisation

The structural development of AM is influenced by soil P concentration (Sanders & Tinker, 1973; Schwab, Menge & Leonard, 1983; Amijee, Tinker & Stribley, 1989; Smith & Gianinazzi-Pearson, 1990; Bolan, 1991; Bruce, Smith & Tester,

1994; Smith & Read, 1997 and many others). The effect of soil P on colonisation interacts with other environmental factors, in particular irradiance (Pearson, *et al.*, 1991; Smith & Read, 1997). Bolan, Robson & Barrow (1984) demonstrated that at low soil P concentrations, colonisation was inhibited, and that small additions of P to the soil increased colonisation slightly. In contrast, larger additions of P to the soil can result in a reduction in colonisation (Smith & Read, 1997 and references therein). The magnitude of the effect of soil P on colonisation differs between plants that have been studied. For example, Baon *et al.* (1992) found that in *Triticum aestivum*, *Hordeum vulgare* and *Secal cereale*, addition of P to the soil could significantly reduce colonisation and almost eliminate it. However, Oliver *et al.* (1983) found that addition of P to a concentration necessary to achieve maximum growth only resulted in a 20% reduction in colonisation of *T. subterraneum*. The reasons for reductions in colonisation with increasing soil P have received a lot of attention. Some experiments have shown that decreases are not in fungal growth but rather are due to increases in the growth of roots (Smith, 1982; Thomson, Robson & Abbott, 1991). Bruce *et al.*, (1994) concluded that reduced colonisation is at least in part due to reduced growth of the fungus in addition to increased root growth.

Effects of soil P concentration on the structural development of AM have also been observed. For example, penetration of roots has been demonstrated to decrease with increased host P nutrition (Schwab *et al.*, 1983). Similarly, Amijee *et al.* (1989) reported a decrease in the number of entry points with increased P

addition in *A. porrum* colonised by *G. mosseae*. Conversely, Thomson, Robson & Abbott (1986) found no decrease in the initial density of entry points formed by *Glomus fasciculatum* and *Scutellospora calospora* colonising *T. subterraneum* with addition of P, but rather a reduction in the amount of colonisation of the root. Bruce *et al.* (1994) observed a reduction in the number of arbuscules but not entry points in *Cucumis sativus* colonised by *G. intraradices* with increasing soil P concentration. Many other workers have also shown that soil P concentration can also influence arbuscular density (Sanders & Tinker, 1973; Amijee *et al.*, 1989; Smith & Gianinazzi-Pearson, 1990; Bruce *et al.*, 1994, see also Chapter 4). From these examples, it is clear that there is considerable variation in the response of AM at the structural level, which may be a reflection of the different plant species, fungal species or experimental conditions used. These issues are considered in more depth in Chapters 4 and 7.

As with most other aspects of AM research, the *Paris*-type has been largely ignored with respect to the effects of colonisation on plant growth and P nutrition. In fact, to my knowledge there have been no specific studies of the effect of *Paris*-type AM colonisation on plant growth, P uptake and nutrition, or effects of P addition on the development of colonisation of this morphological type under controlled conditions.

1.9 Conclusions and aims

Although there are two main morphological types of AM, most research has focused on the *Arum*-type rather than the *Paris*-type. This is largely due to the fact that the majority of crop plant species tend to form the *Arum*-type. Therefore, the aim of the work presented in this thesis was to undertake a detailed study of the *Paris*-type. This was done using a number of different approaches including development of new methods and use of a range of techniques and experimental systems. The broad aims of the work presented in this thesis were therefore:

1. To select a plant and fungal combination which formed the *Paris*-type, suitable for use in glasshouse-based experiments,
2. To quantitatively characterise the time-course of development of a *Paris*-type AM and to develop a method for collection of quantitative information about the relationships between the different fungal structures, hyphal coils, arbusculate coils and external hyphae, over time,
3. To determine if structures formed by *Paris*-type AM result in similar structural modifications in the host plant cells, as observed with *Arum*-type AM,
4. To determine whether *Paris*-type AM are "functional" in terms of improving plant growth and P nutrition over a range of soil P concentrations,
5. To assess what influence soil P concentration has on the morphological development of a *Paris*-type AM,
6. To assess the importance of fungal identity in defining the morphology of AM.

CHAPTER 2 GENERAL MATERIALS AND METHODS

This chapter describes the materials and methods routinely used in this study. Any modifications of these materials and methods are outlined in the relevant chapters.

2.1 Soils

Soils from two sites were used in order to take into account the different pH preferences of the fungal species used. These soils were collected from the Mallala and Kuitpo regions of South Australia. The soils were mixed (individually) with washed river sand (Keogh sands, Australia) to give a 1:9 (w/w) soil/sand mixture for use in experiments. The pH (1:5 soil measured in 0.01 M $CaCl_2$) and bicarbonate-extractable P (modification of Colwell, 1963, see 2.4.2) of the two soils and the soil/sand mixes are given in Table 2.1. All soil and sand was autoclaved at 121° C for 1 h twice over a period of 3 d and then oven dried prior to use.

Table 2.1 pH and bicarbonate-extractable P of the soils and 1:9 (w/w) soil/sand mixes used.

Soil	pH	P concentration (mg P kg^{-1} soil)
Mallala	8.1	16.09
Kuitpo	5.1	14.0
Mallala/sand mix	7.4	6.63
Kuitpo/sand mix	5.3	6.0

2.2 Plant material

2.2.1 Seed sources and germination

Three plant species were used routinely; their identities and sources are given in Table 2.2.

Table 2.2 Plant species used, their authorities and seed source.

Species	Source
Allium porrum L. cv Vertina	S&G Seeds, South Australia (S.A.).
**Asphodelus fistulosus* L.	Collected Ardrossan, S.A., by Dr S. Dickson.
Lycopersicon esculentum Mill. (76r)	Supplied by Ms T. Edmonds, originally obtained from Peto Seed Company, California.

**Asphodulus fistulosus* is referred to as *A. fistulosus* hereafter.

Seed was surface-sterilised prior to use in all experiments. It was placed in a 3% sodium hypochlorite solution for ten min, then in aerated reverse osmosis (RO) water for 20 min and finally placed on filter paper in sealed Petri dishes and stored in the dark at 25° C to germinate.

2.2.2 Watering and nutrient addition

All plants were grown in plastic, non-draining, 400 g or 1.4 kg pots (Polar Cup, Australia). Plants were watered three times a week to 10% (w/w) of soil oven-dry weight. Once a week, starting two weeks after planting of seeds into pots, a modified Long Ashton solution minus P (Appendix 1) was added.

2.2.3 Glasshouse conditions

All plants were grown in a glasshouse on the Waite Campus, Adelaide University, Glen Osmond, South Australia. The mean annual diurnal temperature range was between 22° C day to 14° C night. Light levels were in the range of 250 and 1100 μmol m^{-2} s^{-1} depending on season and weather conditions (D. miller personal communication).

2.3 Fungal material

2.3.1 Fungal isolates

Six fungal isolates were used. The species, authority, source and soil in which they were grown in are given in Table 2.3.

Table 2.3 Fungal species used, their authorities, source and soil in which they were grown.

Fungal species	Authorities	Culture number	Source of isolate	Soil
Gigaspora margarita	Becker & Hall	Supplied prior to establishment of BEG collection	Dr. V. Gianinazzi-Pearson, INRA, Dijion, France	Kuitpo
Glomus coronatum	Giovannetti	WUM 16	Assoc. Prof. L. K. Abbott, Uni. of Western Australia	Mallala
G. intraradices	Schenck & Smith	DAOM 181602	NPI, Utah, USA	Mallala
G. mosseae	(Nicol. & Gerd.) Gerd. and Trappe	NBR 4-1	Dr P. McGee, Uni Sydney, Australia	Mallala
G. versiforme	(Daniels & Trappe) Berch	Supplied prior to establishment of BEG collection	Dr R. L. Peterson, Uni. Guelph, Canada	Mallala
Scutellospora calospora	(Nicol. & Gerd.) Walker & Sanders	WUM 12(2)	Chris Gazey, Uni. of Western Australia	Kuitpo

2.3.2 Inoculum maintenance and production

All fungal isolates were maintained in either *Al. porrum* or *T. subterraneum* L. cv. Mount Barker pot cultures. Pot cultures were produced by mixing inoculum from existing pot cultures with the appropriate soil/sand mix (see Table 2.3) in a 1:9 (w/w) ratio, giving 1.4 kg pot cultures. Pot cultures were grown for four months under the same conditions as all other plants (sections 2.2.2 & 2.2.3). Only pots containing plants where the mycorrhizal colonisation exceeded 70% were used for inoculum in experiments and in culture propagation.

2.3.3 Nurse-pot production

Nurse-pots were produced using a modification (Rosewarne *et al.*, 1997) of the nurse-pot method (Brundrett *et al.*, 1985) as follows: three pre-germinated *Al. porrum* seeds were planted in a triangular arrangement into 70 mm diameter, plastic, non-draining pots (Polar Cup, Australia) containing 400 g of a 1:9 (w/w) mixture of inoculum (as in section 2.3.2) and soil/sand mix (as in section 2.1). Nurse-pots were grown under the same conditions as all other plants (as in sections 2.2.2 and 2.2.3) for 42 d prior to use. Experimental plants were transplanted into the middle of nurse-pots. The typical arrangement of nurse-plants and experimental plants is given in Figure 2.1. The method of establishment of experimental plants differed between chapters and is therefore, presented in the relevant chapters.

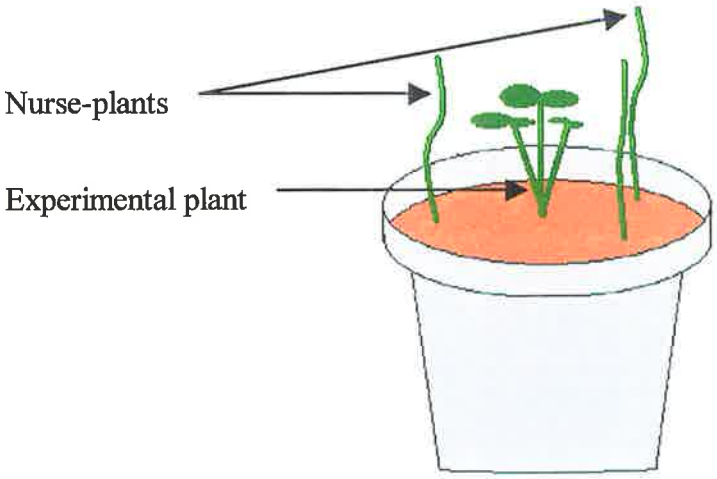


Figure 2.1 Typical arrangement of nurse-plants and experimental plants in a nurse-pot.

2.4 General methods

2.4.1 Harvesting

Plants were harvested by carefully washing them free from the soil with RO water. Once the plants were removed from the pot, any remaining soil and attached debris were washed off. The roots and shoots were separated and fresh weights determined. The shoots and a weighed sub-sample of the roots (when

required/possible) were dried at 80° C for 24 h and the dry weights determined. A weighed sub-sample was cleared and stained as described in section 2.4.4.

2.4.2 Soil phosphorus determination

Soil P was determined using a modification of the method of Colwell (1963) as follows: One gram of oven-dried soil was incubated for 16 h at 20° C in an end-over-end shaker with 100 ml 0.5 M $NaHCO_3$ (pH 8.5). The resulting suspension was then passed through a Whatman No. 42 filter paper to remove large solids. 2.5 ml of the filtrate was neutralised with 2.5 ml 0.5 M HCl. Once this solution stopped effervescing (overnight), 1 ml of the neutralised extract was added to 8 ml of water. To this, 2 ml of freshly prepared Reagent B (see below) was added and mixed. The solution was then incubated at room temperature for 1 h and the P content determined colorimetrically at 830 nm. Reagent B was made as follows: 1 g of ascorbic acid was dissolved in 200 ml of Reagent A. Reagent A was made as follows: 12 g of ammonium molybdate was dissolved in 250 ml of RO water. 0.29 g of antimony potassium tartrate was dissolved in a further 240 ml of RO water. These two solutions were added to 1 l of 5N H_2SO_4 and made up to 2.5 l with RO water.

2.4.3 Plant tissue phosphorus determination

The concentration of P in plant tissues was determined using the method of Hanson (1950) as follows:

Nitric-perchloric digestion

1. Up to 50 mg of oven dry plant tissue was weighed into a 50 ml digestion tube.
2. 3 ml of nitric-perchloric acid (Appendix 2) was added to the tubes and allowed to stand in a fume hood overnight.
3. The tubes were placed in a digestion block programmed as shown in Table 2.4.
4. During the digestion process there is a loss of nitric acid followed by the vigorous perchloric acid reaction. The digestion tubes were removed from the digestion block when the tubes had dried.

Table 2.4 Programmed steps for the digestion of plant tissue.

Step	Temperature (°C)	Ramp (min)	Time (min)
1	150	20	240
2	200	10	60
3	225	10	120

Determination of P in plant material:

1. The digests were made up to 25 ml using RO water.

2. 5 ml aliquots of the diluted digest were taken, to which 3 ml of the colour reagent (Appendix 3) was added and the volume was made up to 25 ml with RO water.
3. After 30 min, the absorbance was read on a spectrophotometer at 390 nm using a blue filter.

2.4.4 Clearing and staining of roots

Roots were cleared and stained using a modification of the method of Phillips & Hayman (1970). Root material was cut into 10 mm lengths and cleared by immersion in 10% KOH at room temperature for 5 d for *A. fistulosus* and *Al. porrum* and 7 d for *Ly. esculentum*. The roots were then rinsed in RO water, immersed in 0.1 M HCl for 20 s and then stained using trypan blue (0.06% in lactophenol) by immersion at room temperature for 2.5 h for *A. fistulosus*, 1 h for *Al. porrum* and 15 min at 80° C for *Ly. esculentum*. The roots were rinsed again with RO water and stored and mounted in 1:1 (v/v) lactic acid and glycerol.

2.4.5 Assessment of colonisation

2.4.5.1 Grid line intersect method

The total root length and root length colonised were determined using the grid line intersect method (Giovannetti & Mosse, 1980) as follows. Stained root material

was transferred to a Petri dish and spread randomly, avoiding overlapping of the roots where possible. The underside of the Petri dish was marked with a grid. The roots were observed at x 40 magnification and the total number of times root pieces crossed the grid lines and the number of times colonised sections of root crossed the grid lines were scored. These two values were used to calculate the percent colonisation of the roots. The total root length and the mycorrhizal root length was calculated from the total or colonised intersects using the following equation:

Length = number of intersects x G x 11/14

Where G is the length of one side of a grid square (10 mm in this case).

2.4.5.2 Magnified Intersects Technique

The magnified intersects technique of McGonigle *et al.* (1990) was used to determine the fraction of the root colonised by different structures. Root pieces were mounted in five parallel rows on microscope slides and observed at x 160 magnification. Fifty observations were made for each of two slides per plant, a total of 100 intersections. A cross-hair in the eyepiece of the microscope was aligned to be parallel with the surface of the root when the centre of the cross-hair intersected a root (see Figure 2.2). At each intersection the presence of AM structures (depending on the morphological type of AM being observed) and intersections containing no fungal structures were scored and recorded. These

data were then used to calculate the fractional colonisation by each of the structures. In this study, external hyphae (EH) were defined as those hyphae attached to the surface of the root leading back to a penetration point. Other structures scored were hyphal coils (HC), arbusculate coils (AC), intercellular hyphae (IH), arbuscules (A), hypodermal entry coils (EC) and vesicles (V).

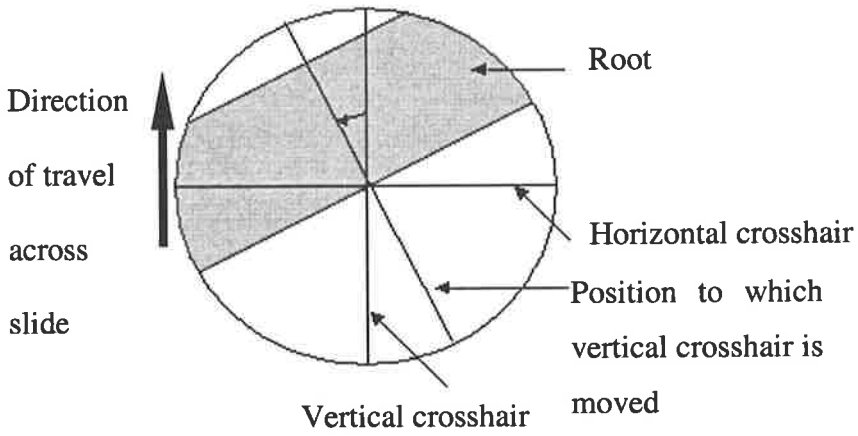


Figure 2.2 Rotation of the vertical crosshair to ensure that the scoring of structures is along a line perpendicular to the long axis of the root.

2.4.6 Sectioning of plant material

Plant material was sectioned using a modification of the methods of Smith & Dickson (1991). Roots were embedded in gelatin blocks consisting of 10% gelatin and a drop of glycerol made up in RO water. The gelatin blocks were frozen on to a Frigistor stage and sectioned with a freezing microtome (Leitz,

Germany). Longitudinal, 120 μm sections were cut and transferred directly into the desired stain.

2.4.7 Statistical analysis

ANOVA was performed on all data using Genstat 5 Release 4.1 (1998). Where significant differences were found, differences between treatment means were calculated using the least significant differences (LSD) method.

CHAPTER 3 SELECTION OF A *PARIS*-TYPE AM

3.1 Introduction

The main aim of the work described in this thesis was to increase our understanding of *Paris*-type AM, and if possible to determine what role interfaces aside from arbuscules have in nutrient transfer processes.

Much of the laboratory based research on AM has been conducted using "model" plant species, for example, *Al. porrum* (Brundrett *et al.*, 1985; Ezawa, Saito & Yoshida, 1995; Dickson, Smith & Smith, 1999a, b and many others) and *T. subterraneum* (Abbott, 1982; Wilson, 1984; Bürkert & Robson, 1994; Facelli *et al.*, 1999a, b and many others), both of which form the *Arum*-type. The use of model plants allows for comparison between different experiments. However, there are no laboratory based "model" *Paris*-type AM, with most work on this type coming from field collected material, for example, *Ac. saccharum* (Yawney & Schultz, 1990; Cooke *et al.*, 1993; Moutoglis *et al.*, 1995) and *Pa. quinquefolius* (Whitbread, McGonigle & Peterson, 1996; McGonigle *et al.*, 1999). Thus, the first step in this research was to find a plant/fungus combination which formed a *Paris*-type AM suitable for laboratory based studies.

There have been reports that some *Paris*-type AM lack arbuscules. AM which do not form arbuscules, but only hyphal coils (*Paris*-type), or only

intercellular hyphae (*Arum*-type), will be useful in determining the importance of interfaces aside from arbuscules and arbusculate coils in nutrient transfer processes (Smith & Smith, 1997). Reports of AM without arbuscules come from field-collected material, rather than from experiments conducted under controlled conditions. Therefore, the occurrence of AM which lack arbuscules needs to be verified in the laboratory.

Specifically, the aims of the work presented in this chapter were therefore:

I. To select a plant/fungus combination which formed a *Paris*-type AM, for use in further experiments by inoculating a range of plant species with known fungal species under controlled conditions and to quantitatively assess the morphology of colonisation,

II. To identify, if possible, an AM which did not have an arbuscular phase of colonisation.

In parallel with the experiment presented here, an experiment assessing the morphology of AM formed by *Al. porrum* and *Scutellospora calospora* at a range of soil P concentrations was conducted. This experiment was based on the results of an unpublished experiment where arbuscules were not formed in *Al. porrum* colonised by *S. calospora* (S. Ayling, personal communications). Although this experiment was conducted as part of the search for a suitable *Paris*-type AM, the complicated nature of the results meant that it was more appropriate to present them separately in Chapter 4.

3.2 Materials and methods

3.2.1 Plant species and germination

Based on reports of their morphology in the literature (including Smith & Smith, 1997), nine plant species were selected for assessment of their morphological development. The species were: *Viola cornuta* L. (referred to as *V. cornuta* hereafter) (S&G Seeds, South Australia), *Linum usitatissimum* L. (referred to as *L. usitatissimum* hereafter) (Collected from Clare, South Australia by Dr B. Thomas), *Petroselinum crispum* Hill (referred to as *P. crispum* hereafter) (Yates Seeds, Australia), *A. fistulosus* (see section 2.2.1), *Acer palmatum* Marsh (referred to as *Ac. palmatum* hereafter) (collected from Aldgate, South Australia by Prof. S. E. Smith), *C. rotundus* L. (collected from Moree, New South Wales by Dr E. de Silva), *Exacum affina* Balf. (referred to as *E. affina* hereafter) (S&G Seeds, South Australia), *Solanum centrale* Black (referred to as *S. centrale* hereafter) (Nindethana Seed Service, Western Australia) and *Paris quadrifolia* L. (B & T World Seeds, UK). Germination of the seeds was attempted as follows:

Seeds of *V. cornuta L. usitatissimum*, *P. crispum*, *A. fistulosus*, *E. affina* and *S. centrale* were all surface sterilised and imbibed in RO water as described in section 2.2.1. The seeds were then placed on filter paper in sealed Petri dishes in the dark at 25° C and checked daily for up to 21 d to assess germination.

Ac. palmatum seed was treated as follows: the seed was surface sterilised with 3% sodium hypochlorite for 10 min after removal of the "seed-wings". It was then placed in water and incubated at 45° C for 48 h and finally, was placed in a bag containing moist, autoclaved vermiculite and stored at 4° C. After 4 months germination was assessed on a weekly basis.

Paris quadrifolia seeds were surface sterilised with 3% sodium hypochlorite for 10 min and then imbibed in water for 10 min, stored for 3 months at 20° C, then 5° C for another 3 months and finally at 20° C for a further 3 months (R. Koide, personal communication). Seeds were then placed on moist filter paper in sealed Petri dishes in the dark at 25° C and checked daily for up to 28 d to assess germination.

C. rotundus does not grow from seed, but rather from a tuber. Prior to planting, the tubers were surface sterilised with 3% sodium hypochlorite for 10 min and then rinsed thoroughly with RO water to remove any traces of the sodium hypochlorite.

After germination, the *L. usitatissimum*, *V. cornuta*, *P. crispum* and *A. fistulosus*, seedlings were all planted into 1.4 kg pots containing soil/sand mix inoculated with either *Glomus coronatum* or *Scutellospora calospora*, as in section 2.3.1. The plants were watered and nutrients were applied as in section 2.2.2.

Pre-germinated *E. affina* and *Ac. palmatum* seeds were planted into pots containing inoculum (as above); however, the plants failed to grow. Therefore, the germinated seeds were planted in autoclaved UC mix to allow for growth for six weeks prior to transplanting into pots containing soil/sand mix inoculated with either *G. coronatum* or *S. calospora*. Again both species died when planted into the soil/inoculum mix. Given the slow and unsuccessful growth, and success with other species (see results), further investigations were not undertaken with these two species.

After repeated attempts, *Paris quadrifolia* and *S. centrale* did not germinate. Therefore, these species are not discussed further.

3.2.2 Timing of harvests

All plants were harvested and the growth assessed as in section 2.4.1, and the roots were cleared and stained as in section 2.4.4. For each AM the proportions of the roots containing external hyphae (EH), intercellular hyphae (IH), arbuscules (A), hyphal coils (HC) and arbusculate coils (AC) were scored as in section 2.4.5.2. *L. usitatissimum* and *V. cornuta* plants were harvested at 2, 4 and 6 weeks after planting. *P. crispum* and *A. fistulosus* were harvested 2 and 4 weeks after planting. *C. rotundus* plants were harvested 3 weeks after planting.

3.3 Results

Assessment of colonisation and determination of AM morphological type using the method of McGonigle *et al.* (1990) was successful. The results for each plant species are presented separately below. Since the main aim of this experiment was to select a plant on a morphological basis, growth data are referred to briefly and presented in Appendices 4 to 7.

3.3.1 *Linum usitatissimum*

The percentage of the root length of *L. usitatissimum* colonised differed with both fungal species and time (Figure 3.1 a). Both fungal species formed EH, HC, IH and A, with clear quantitative differences between the two fungi and harvest times (see Figures 3.1 b-e). The colonisation pattern was variable, with some regions of the root containing predominantly HC within the cortex of the root, whereas other regions contained predominantly IH and A, thus *L. usitatissimum* was classified as forming an intermediate type AM. At the four week harvest, with both fungi, there was a large decrease in the percentage of the colonised root length containing A.

The growth data, SDW and RDW, for *L. usitatissimum* are presented in Appendix 4. In general terms, inoculation of *L. usitatissimum* with *S. calospora*

and *G. coronatum* resulted in an increase in the SDW by the 4 and 6 week harvests respectively. Plants colonised by *S. calospora* had a significantly higher SDW than those colonised by *G. coronatum* at the 2 and 6 week harvests and the reverse at the 4 week harvest.

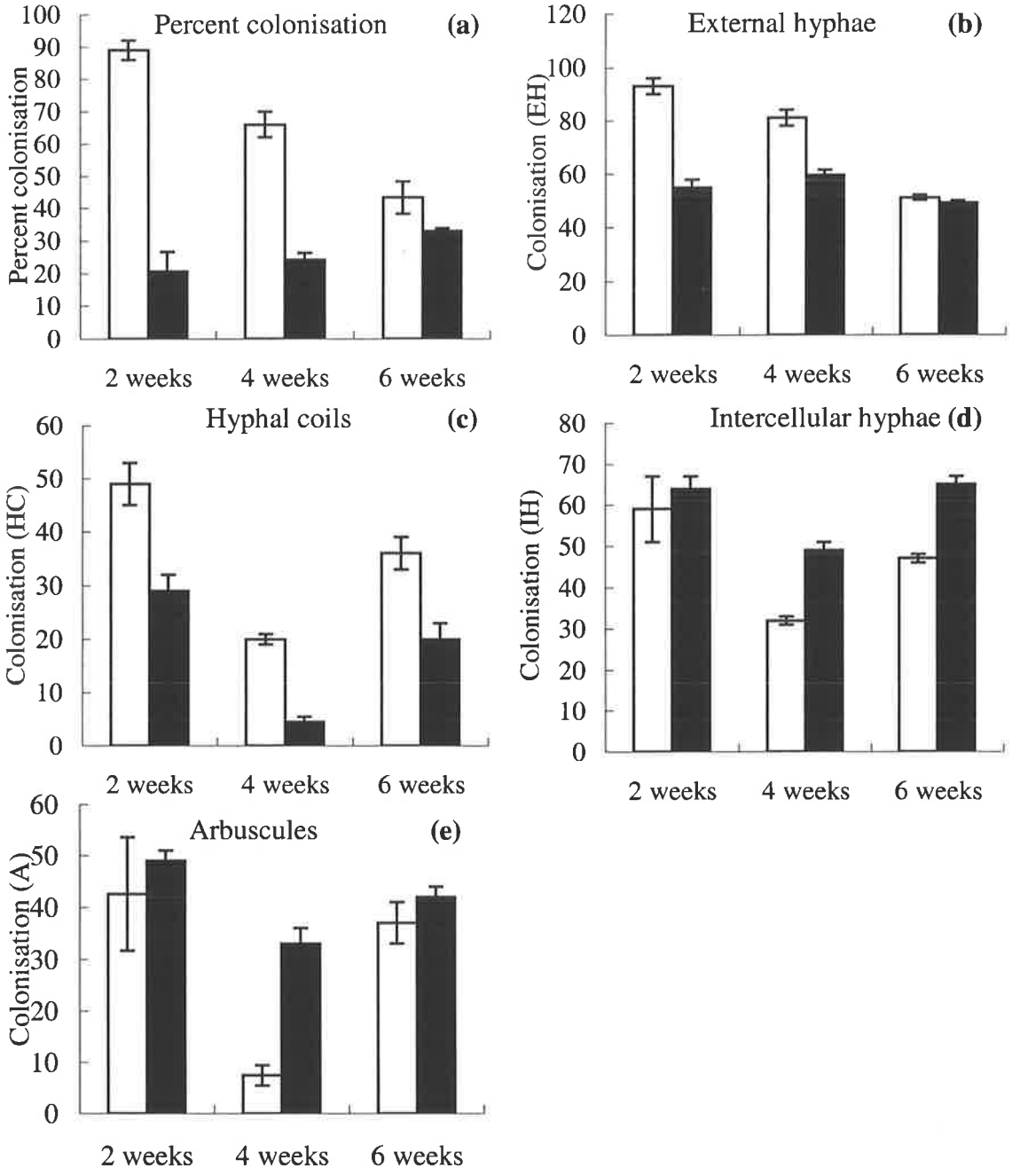


Figure 3.1 *L. usitatissimum* colonised by *G. coronatum* (□) and *S. calospora* (■). (a) Total percentage of the root length colonised and the percentage of the colonised root length containing (b) external hyphae (EH), (c) hyphal coils (HC), (d) intercellular hyphae (IH), (e) arbuscules (A). Values are means +/- S.E. (n=4).

3.3.2 *Viola cornuta*

Both fungal species and time affected the percentage of the root length of *V. cornuta* colonised (Figure 3.2 a). EH, HC (not entry coils in the hypodermis), IH and A were formed by both fungi at all harvest times (see Figure 3.2 b-e), thus *V. cornuta* was classified as forming an intermediate type AM. The colonisation pattern was uniform, with the fungi tending to form HC, IH and A evenly throughout the cortex of the root.

Plants colonised by *G. coronatum* showed a positive growth response (SDW) after 4 weeks, as did the plants colonised by *S. calospora* (see Appendix 5).

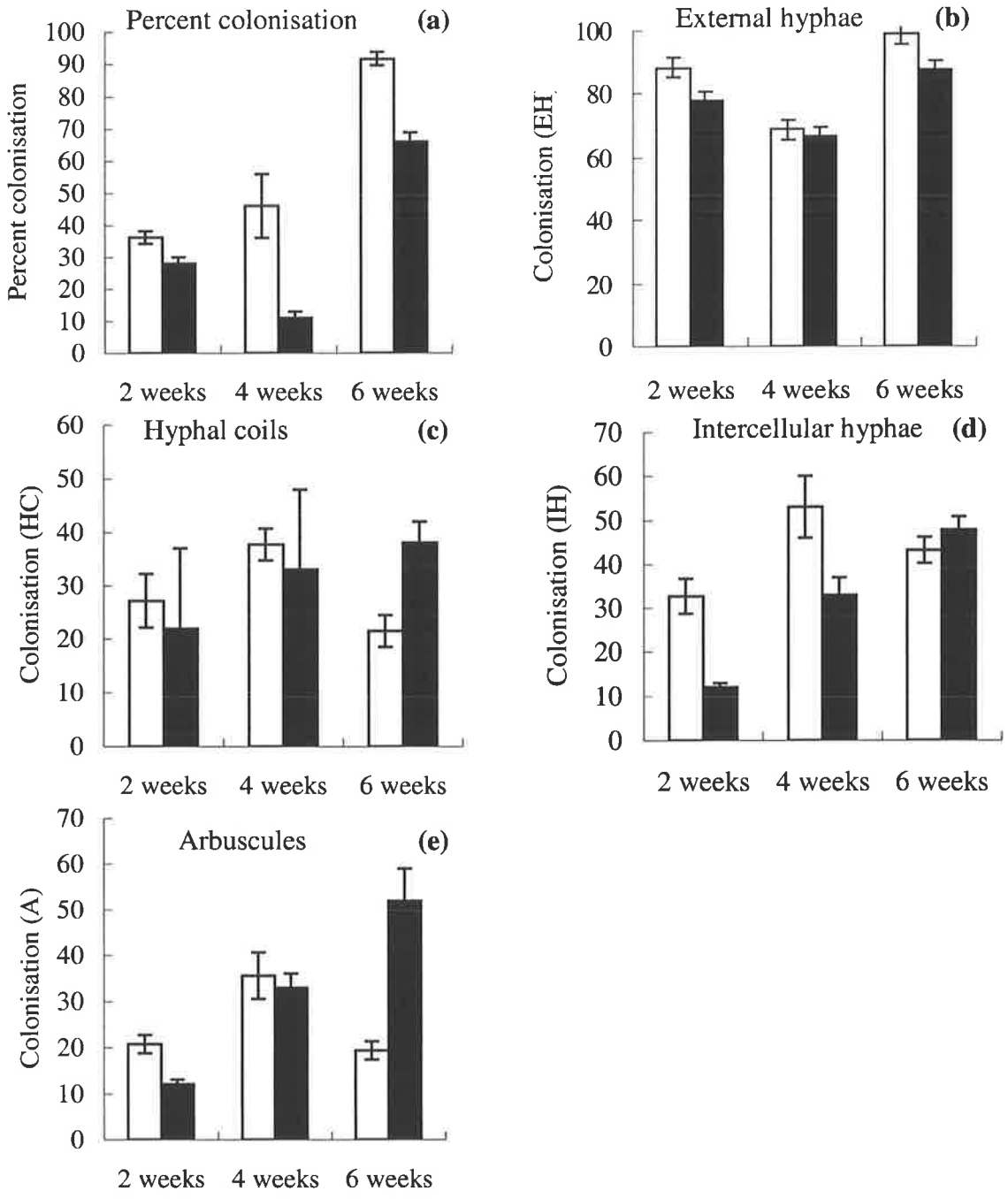


Figure 3.2 *V. cortuna* colonised by *G. coronatum* (□) and *S. calospora* (■). (a) Total percentage of the root length colonised and the percentage of the colonised root length containing (b) external hyphae (EH), (c) hyphal coils (HC), (d) intercellular hyphae (IH), (e) arbuscules (A). Values are means +/- S.E. (n=4).

3.3.3 *Petroselenum crispum*

At the 2 week harvest the percentage of the root length of *P. crispum* colonised was less than 10%. Some hyphae were attached to the surface of the roots, but there was very little internal colonisation (data not shown). At the 4 week harvest *P. crispum* was colonised by both *S. calospora* and *G. coronatum*. Both of the fungi formed EH on the surface of the roots and HC and AC within the cortex of the roots (Figure 3.3). There were no IH, thus, *P. crispum* was classified as forming a *Paris*-type AM. Colonisation of *P. crispum* by *S. calospora* resulted in a slight increase in SDW and colonisation by *G. coronatum* had no effect on SDW at the 4 week harvest (see Appendix 6).

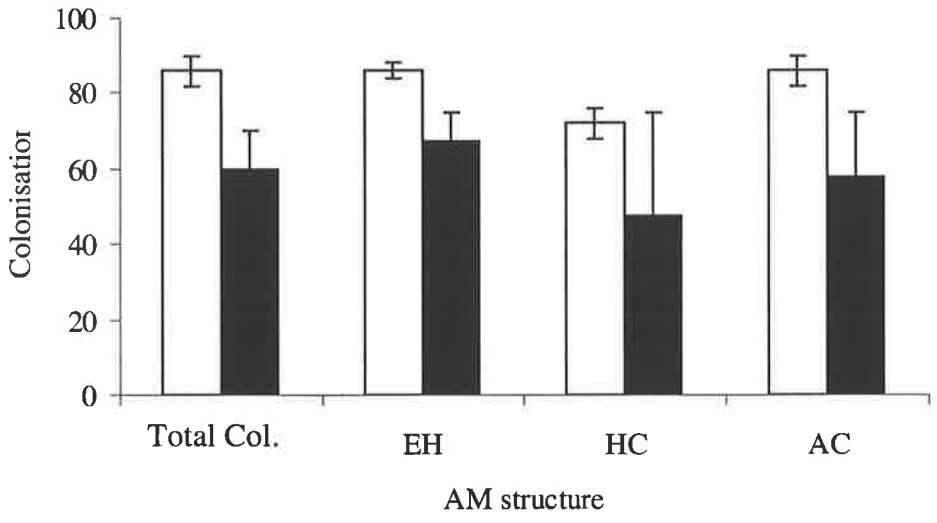


Figure 3.3 *P. crispum* colonised by *G. coronatum* (□) and *S. calospora* (■). Percentage of the total root length colonised (Total col.) and percentage of the colonised root with external hyphae (EH), hyphal coils (HC) and arbusculate coils (AC). Values are means +/- S.E. (n=4).

3.3.4 *Asphodelus fistulosus*

As with *P. crispum*, at the 2 week harvest the percentage of the *A. fistulosus* root length colonised was very low and restricted to hyphae attached to the surface of the roots (data not shown). At the four week harvest, *A. fistulosus* was colonised by both *S. calospora* and *G. coronatum* (Figure 3.4). Both fungi formed EH on the surface of the roots and HC and AC within the cortex of the roots (Figure 3.4), but no IH. Thus *A. fistulosus* was classified as forming a *Paris*-type AM. Colonisation of *A. fistulosus* by *G. coronatum* and *S. calospora* resulted in increased SDW and a slight decrease in the RDW of the plants colonised by *S. calospora*, at the 4 week harvest (see Appendix 7).

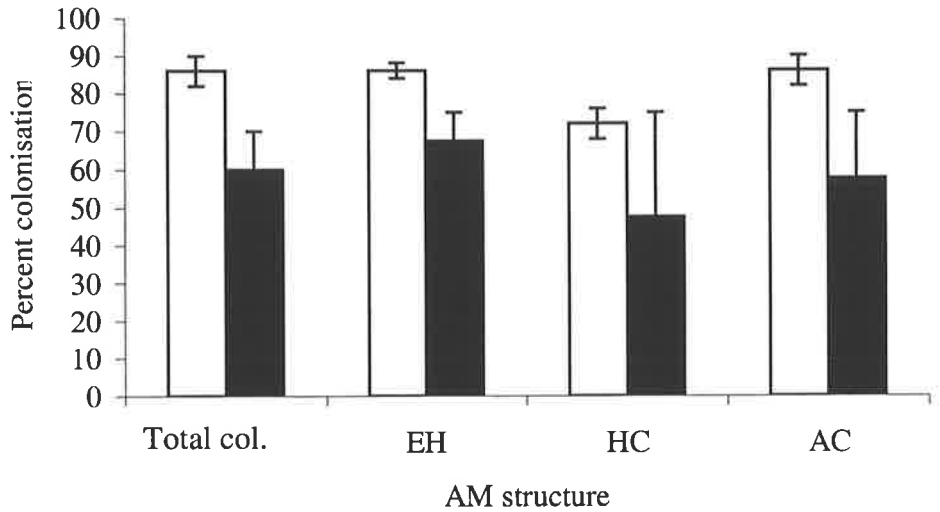


Figure 3.4 *A. fistulosus* colonised by *G. coronatum* (□) and *S. calospora* (■). Percentage of the total root length colonised (Percent col) and percentage of the colonised root with external hyphae (EH), hyphal coils (HC) and arbusculate coils (AC). Values are means +/- S.E. (n=4).

3.3.5 *Cyperus rotundus*

C. rotundus was not readily colonised by either of the fungi under the conditions of the experiment. There were appressoria on the surface of the roots, but, the colonisation did not proceed past this point.

3.4 DISCUSSION

The main aim of the work presented in this chapter was to identify a plant and fungal combination which formed a *Paris*-type AM for use in further experiments. This was attempted by growing a range of plant species with two AM fungi and assessing their morphology quantitatively using the method of McGonigle *et al.* (1990). The results show that this method could be used to demonstrate that clear morphological differences occurred between different treatments, as has been shown previously (Muthukumar *et al.*, 1996; Rosewarne *et al.*, 1997; van der Heijden *et al.*, 1998a).

A range of AM morphological types were formed by the different plant species. *V. cornuta* and *L. usitatissimum* formed intermediate type AM, whereas *P. crispum* and *A. fistulosus* formed *Paris*-type AM. The formation of an intermediate AM morphology by *L. usitatissimum* was unexpected as *L. usitatissimum* has been reported to form the *Paris*-type (Stelz, 1968; B. Thomas,

personal communication) and another member of the Linaceae, *L. catharticum*, is reported to form the *Paris*-type (Gay *et al.*, 1982). The morphology of AM formed by V. *cornuta* is not clear in the literature. Stelz (1968) and Gallaud (1905) both reported that the Violaceae form *Paris* type AM, whereas Widden, DeBellis & Semeniuk (1999) (after the time these experiments were conducted) reported *V. cornuta* as forming characteristics of both types of AM. The results presented here concur with those of Widden *et al.* (1999). Smith & Smith (1997) noted that there are some examples where a single plant species have been reported to form different morphological types of AM in different experiments. This may be related to the species of fungi colonising the roots, the importance of which is considered in Chapter 8.

C. rotundus is a member of the Cyperaceae, a largely non-mycorrhizal family However, Muthukumar *et al.* (1997) reported that *C. rotundus* was colonised by AM fungi and formed intercellular hyphae and vesicles, but that arbuscules were absent. Therefore, it was included in this experiment as it may provide insight into the importance of arbuscules in AM nutrient transfer processes. However, *C. rotundus* was not colonised in this experiment. This may be due to the timing of the harvest, species of fungi or the environmental conditions. *C. rotundus* grows from a tuber, and is very difficult to work with as the timing of the sprouting of the tuber is not reliable. Thus, I did not persist with this species.

P. crispum formed a *Paris*-type AM with both HC and AC, consistent with the reports of Söderström *et al.* (1996). As with *L. usitatissimum* and *V. cornuta*, there were some differences in the proportion of the colonised root containing the different structures between the two fungi when colonising *P. crispum*. One disadvantage of using *P. crispum* is that after clearing and staining the roots became brittle and fell apart. Thus, they are not ideal for use in further experiments.

A. fistulosus formed a *Paris*-type AM with clearly distinguishable HC and AC with both fungi. This is consistent with observations of field collected material (Cavagnaro, unpublished results). Interestingly, on the basis of the distribution of *Arum*- and *Paris*-type AM in the liliaceae shown by Smith & Smith (1997), I would have expected *A. fistulosus* to form the *Arum*-type. However, the report of the Asphodellaceae forming the *Arum*-type is based on observations of *Aloe* (Gallaud, 1905), which has a very different root form from *A. fistulosus*. This highlights the complexity of the basis of the two classes of AM. Unlike *P. crispum*, the roots were easy to handle and relatively simple to clear and stain. The density of external hyphae formed on the root surface by *G. coronatum* was lower than that of *S. calospora*. Thus, it was easier to observe the internal morphology of the AM formed when roots were colonised by *G. coronatum*. *A. fistulosus* has an endodermis, however, coils are not formed in this cell layer, thus, the coils in *A. fistulosus* are true cortical coils and not entry coils. When colonised by either of the fungi, *A. fistulosus* showed an increase in SDW at the 4

week harvest. Therefore, on the basis of the plant species tested, *A. fistulosus* was the most suitable for use in further experiments.

There were differences in the morphology of AM formed in this experiment compared to published reports. Many studies of AM morphology have been made using field collected material, where the identity of the AMF is not known. Further complications with field-collected material might include unknown age, varied environmental conditions such as nutrient availability, soil type, light, etc. The results presented here showed clear quantitative differences in the AM morphology between the different AMF. For example, *L. usitatissimum* showed considerable differences in the relative frequency of the different structures, between the two fungi tested. Such differences in morphology have been noted in other experiments and may be related to fungal identity. For example, as outlined in Chapter 1, Abbott (1982) developed a key to identify the fungal species colonising the roots of *T. subterraneum* based on the internal morphology of colonisation for ten different Glomalean fungi. Variation in the morphology of AM associations formed by *Daucus carota* (carrot) have been observed (Söderström *et al.*, 1996) with different fungi. The importance of such studies with respect to possible effects on AM morphology is further considered, in Chapter 8.

All of the AMF investigated in this chapter formed either arbuscules and arbusculate coils. The reports of an absence of arbuscules are largely from field

based studies. The occurrence of AM which do not form arbuscules may be important with respect to function (Smith & Smith, 1997). However, it would have been very time consuming to search for a possible "arbuscule-free" plant and to verify the lack of arbuscules under a wide range of conditions in the laboratory. Thus I decided not to persist with this aspect of research, but rather focused on the development and functioning of *Paris*-type AM.

In conclusion, *A. fistulosus* forms a *Paris*-type AM with both *G. coronatum* and *S. calospora*. The internal morphology of roots colonised by *G. coronatum* was clearer than those colonised by *S. calsopora*. Thus, the AM formed by *A. fistulosus* and *G. coronatum* was selected for use in the majority of experiments presented in this thesis.

CHAPTER 4 EFFECT OF PHOSPHORUS ON THE DEVELOPMENT OF THE *ALLIUM PORRUM*/ *SCUTELLOSPORA CALOSPORA* SYMBIOSIS

4.1 Introduction

The results in this chapter were presented in a preliminary form at the First National Conference on Mycorrhizas, Bogor, Indonesia.

Cavagnaro TR, Smith SE, Smith FA, Ayling SM. 1999. The effect of P addition on growth of Leek (*Al. porrum* L.) and its fractional colonisation by *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders. *First National Conference on Mycorrhizas, Bogor, Indonesia, conference proceedings.*

Al. porrum forms an *Arum*-type AM morphology, with characteristic intercellular hyphae and arbuscules, when colonised by a range of AMF. These fungi include *Glomus versiforme*, *Scutellospora calospora* and *G. coronatum* (Brundrett *et al.*, 1985; Ezawa *et al.*, 1995; Smith & Smith, 1997; Dickson *et al.*, 1999a, b and many others). However, in an unpublished experiment (S. Ayling, personal communications), arbuscules were not formed in *Al. porrum* colonised by *S. calospora*. This conflicts with previous reports.

Dr Ayling grew her plants under the same conditions and in the same glasshouse in which I did my experiments. Therefore, I hoped to be able to reproduce her results. If the reported absence of arbuscules can be reproduced, then the *Al. porrum/S. calospora* symbiosis would provide a useful system to study the importance of interfaces other than arbuscules in the functioning of AM, especially with respect to P transfer.

Both the extent and morphology of colonisation of plant roots by AMF are influenced by abiotic and biotic factors (Jacquelinet-Jeanmougin & Gianinazzi-Pearson, 1983; Cooke *et al.*, 1993; Braunberger e*t al.*, 1991; Smith & Smith, 1997). Effects of soil P concentration on the morphology of AM have been widely studied, showing varied effects on the production of entry points and arbuscules (for example, Amijee *et al.*, 1989; Bruce *et al.*, 1994; Smith & Gianinazzi-Pearson, 1990). Given that soil P concentration can influence the morphological development of AM, it may be useful to investigate these effects on the *Al. porrum/S. calospora* symbiosis.

When assessing the effects of P on the morphological development of AM, different methods of data collection and presentation have often been used. For example, most researches have expressed results as arbuscules per unit root length. However, a reduction in overall colonisation would show a reduction in arbuscules per root length rather than there necessarily being a direct effect on arbuscules within the colonised regions of the roots. Therefore, when comparing

experiments this issue should be taken into careful consideration (see also Chapter 5).

The aims of the work presented in this chapter were therefore:

I. To reproduce conditions under which *S. calospora* is reported to colonise *Al. porrum* without arbuscule formation,

II. To determine if P addition to the soil and harvest time have an effect on the growth and morphological development (expressed as a percentage of colonised root length) of this symbiosis.

4.2 Materials and methods

4.2.1 Plant growth

Al. porrum seed was surface sterilised, pre-germinated (section 2.2.1) and planted in 70 mm diameter, plastic, non-draining pots containing 400 g of the inoculum/Kuitpo soil/sand mix (section 2.3.1). Prior to planting the germinated *Al. porrum* seeds, the P content of the inoculum and soil/sand mix was altered by the addition of $NaH_2PO_4.H_2O$ solutions, giving final soil P concentrations of 6 (no added P), 14 and 31 mg $P.kg^{-1}$ soil and referred to as P1, P2, and P3 respectively. Plants were grown in a glasshouse, were watered 3 times a week and nutrients were added weekly (section 2.2.2).

The plants were harvested (section 2.4.1) at 2, 4 and 6 weeks after planting. At each harvest time the shoot dry weights and root fresh weights were determined. Root material was cleared and stained (see section 2.4.4) and colonisation was assessed using the magnified intersects technique (see section 2.4.5.2). The percentages of the colonised root length containing arbuscules (A), entry coils (EC), intercellular hyphae (IH) and external hyphae (EH) were scored. In this experiment I have expressed arbuscule and entry point numbers as a percentage of the colonised root length, thus allowing for differentiation between effects on individual structures and colonisation as a whole.

4.3 Results

4.3.1 Colonisation

Al. porrum colonised by *S. calospora* formed an *Arum*-type AM, with characteristic EC in the hypodermis, IH and A. The percentage of the root length colonised and the percentage of the colonised root length containing EC, IH and AR are presented in Table 4.1. Due to the complex statistical interactions found between the different treatments (harvest time, inoculation with *S. calospora* and soil P concentration), details of these interactions are given in separate tables and referred to where appropriate.

Table 4.1 Summary of root colonisation data for all harvest times and soil P concentrations. Values are the mean percentage of the root length colonised, and percentage of the colonised root length containing either EC, IH or A. (n=4).

		2 weeks	4 weeks	6 weeks
P1	% colonisation	15.7	84.7	89.7
	Entry coils	0	21.3	28.7
	Intercellular hyphae	0	66.3	79.3
	Arbuscules	0	21.7	28.3
P2	% colonisation	10.7	86.7	83.3
	Entry coils	0	22.3	27.7
	Intercellular hyphae	0	61.9	74.3
	Arbuscules	0	15.0	22.0
P3	% colonisation	9.0	81.0	76.7
	Entry coils	0	23.3	27.7
	Intercellular hyphae	0	72.4	67.3
	Arbuscules	0	12.3	17.3

The percentage of the root length colonised by *S. calospora* was influenced significantly by harvest time and soil P concentration. However, there was no significant 2-way interaction between harvest time and soil P concentration. At the 2 week harvest there was 9 to 15% colonisation by *S. calospora* (Table 4.1), made up largely of EH attached to the surface of roots by appressoria, but no EC, AR or IH were scored. Between the 2 and 4 week harvests there was a significant increase in colonisation, but no further increase thereafter (Table 4.2). P addition significantly reduced colonisation between the P2 and P3 treatments (Table 4.2), but not between the P1 and P2 treatments.

Table 4.2 Percentage of root length colonised at 2, 4 and 6 week harvests and three soil P concentrations. Means followed by the same letter are not significantly different at the 0.05 level. (n=4).

HARVEST (weeks)	**2**	**4**	**6**
Percent colonisation	11.8^{a}	84.1^{b}	83.2^{b}
P CONCENTRATION (mg. kg^{-1})	**P1**	**P2**	**P3**
Percent colonisation	63.3^{a}	60.2^{a}	55.6^{b}

Due to the low level of colonisation at the 2 week harvest (11.8%), data from this harvest have been excluded from analysis of EC and A formation. Again there was no 2-way interaction between harvest time and soil P concentration on the percentage of the colonised root length containing EC. Therefore, only the main effects are presented. Soil P concentration did not have an effect on the number of intersections containing EC, whereas harvest time did (Table 4.3).

Table 4.3 Percent of colonised root length containing EC at 4 and 6 week harvests and three soil P concentrations. Means followed by the same letter are not significantly different at the 0.05 level. (n=4).

HARVEST (weeks)		**4**	**6**
Entry Coils		22.23^{a}	28.0^{b}
P CONCENTRATION	**P1**	**P2**	**P3**
Entry Coils	25.0^{a}	25.0^{a}	25.5^{a}

When considering the percentage of the colonised root length containing A, there was no 2-way interaction between harvest time and soil P concentration. Between the 4 and 6 week harvests there was a significant increase in the fractional arbuscular colonisation (Table 4.4). Conversely, increased soil P concentration resulted in a significant decrease in the percentage of the colonised root length containing A.

Table 4.4 Percent of colonised root length containing A at 4 and 6 week harvests and three soil P concentrations. Means followed by the same letter are not significantly different at the 0.05 level. (n=4).

HARVEST (weeks)		**4**	**6**
Arbuscules		16.3^{a}	22.6^{b}
P CONCENTRATION	**P1**	**P2**	**P3**
Arbuscules	25.0^{a}	18.5^{b}	14.8^{c}

4.3.2 Plant growth

Shoot dry weight data are presented in Figure 4.1. There was a significant three-way interaction between harvest time, inoculation treatment and soil P concentration. Shoot dry weights increased with time. Addition of P to the soil also had a large effect on plant growth at the 6 week harvest but not at the 2 and 4 week harvests. Colonisation of *Al. porrum* by *S. calospora* did not have a significant effect on SDW at the 2 and 4 week harvests, with the exception of P3 at the 4 week harvest where the uncolonised plants were larger than the colonised plants (Figure 4.1). At the 6 week harvest there was a significant positive growth response due to *S. calospora* in the P1 and P2 treatments, but not at P3.

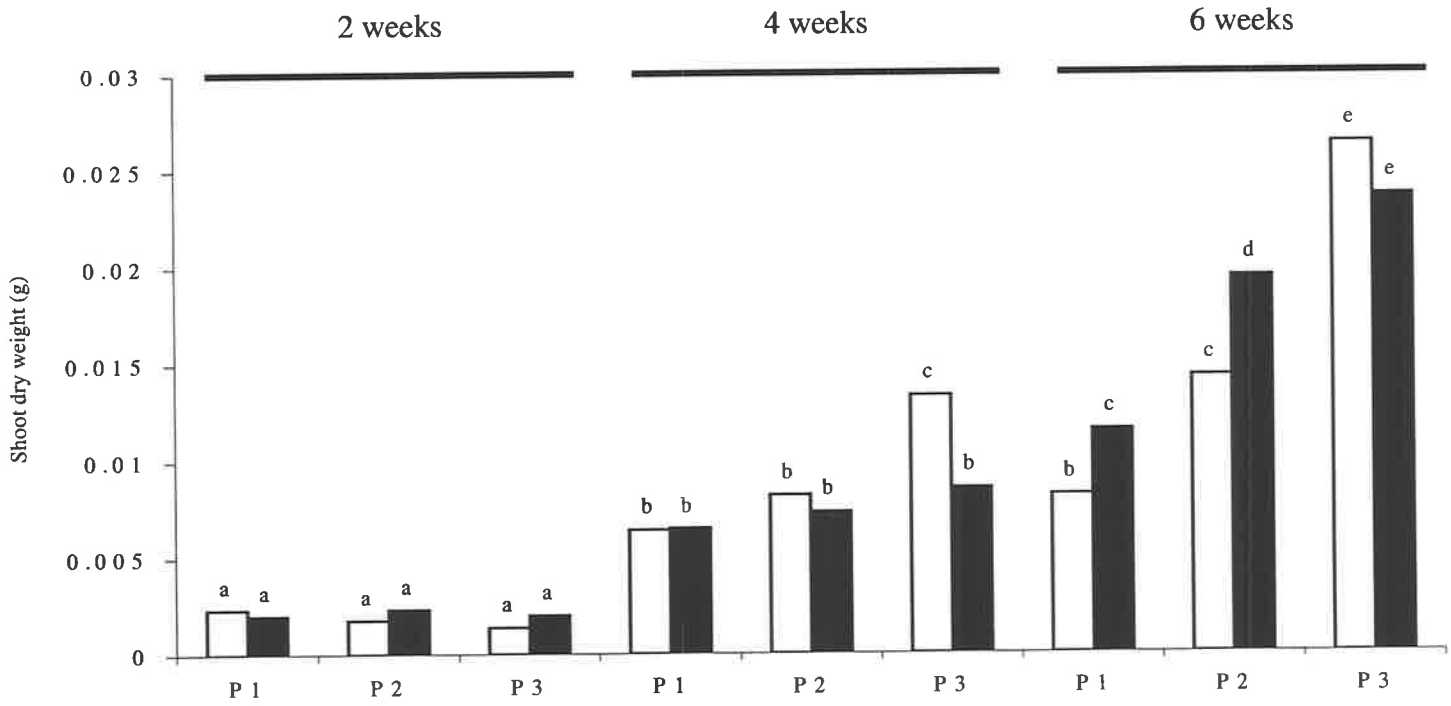


Figure 4.1 Shoot dry weight of *A. porrum* plants not inoculated □ and inoculated ■ with *S. calospora* at three harvest times and three soil P concentrations (P1, P2 and P3). Means followed by the same letter are not significantly different at the 0.05 level. (n=4).

Root fresh weight (RFW) data are presented rather than root dry weight data because there was insufficient root material to accurately measure both root dry weight and fractional colonisation at all harvest times. There was not a significant three-way interaction between soil P concentration, mycorrhizal treatment and harvest time. However, a significant 2-way interaction between harvest time and soil P concentration was found. RFW was not influenced by the addition of P to the soil at the 2 and 4 week harvests, with the exception of P3 at the 4 week harvest where the RFW was significantly higher than that of the other treatments (Table 4.5). At the 6 week harvest, increased soil P concentration resulted in a significant increase in the RFW. Inoculation of the plants with *S. calospora* had no effect on root fresh weight at any harvest time and soil P concentration.

Table 4.5 Root fresh weight of *Al. porrum* at the 2, 4 and 6 week harvests and three soil P concentrations. Means followed by the same letter are not significantly different at the 0.05 level. (n=4).

	P CONCENTRATION	**P1**	**P2**	**P3**
HARVEST (weeks)	**2**	0.047^{a}	0.032^{a}	0.023^{a}
	4	0.075^{b}	0.099^{b}	0.130^{c}
	6	0.131^{c}	0.235^{d}	0.343^{e}

4.4 Discussion

The results of this experiment do not support Dr Ayling's observation that *Al. porrum* colonised by *S. calospora* did not form arbuscules, except at two weeks when total colonisation was very low. The results are in line with previous work in which *S. calospora* produced arbuscules in *Al. porrum* (Dickson *et al.*, 1999a, b). The present experiment and Dr Ayling's experiment were conducted using the same sources of seed, fungi and soil and grown in the same glasshouse, yet there were large differences between the results of the two experiments. These differences remain unexplained and the work was not followed up. Nevertheless, the results are worth discussing with previous relevant work on responses of AM morphology to increased soil P.

4.4.1 Colonisation

Changes in the percentage of the root length colonised by AMF over time are well described (for example, Saif, 1977; Sanders *et al.*, 1977, and many subsequent papers). In the present experiment, there was a six fold increase in the percentage of the root length colonised between the 2 and 4 week harvests (Table 4.1). This increase is similar to the period of rapid increase in colonisation after an initial lag phase in colonisation as described by Sanders *et al.* (1977). The proportion of the root length colonised at 2 weeks was considerably less than that reported by

Dickson *et al.* (1999a), but the values were similar at the 4 week harvest. The difference in the rate of establishment of colonisation in the present experiment and in the work of Dickson *et al.* (1999a) using the same material (see above) may be related to propagule densities in the inoculum, rates of spore germination or other environmental factors (see Sanders *et al.*, 1977; Smith & Read, 1997). Between the 4 and 6 week harvests there was only a small increase in the percentage of the root length colonised, consistent with the results of Dickson *et al.* (1999a). This is also in agreement with the steady state phase of colonisation described by Sanders *et al.* (1977) in which the rates of root growth and fungal spread are the same. The most frequently scored structure in the roots was intercellular hyphae. This is expected since arbuscules and entry coils are generally not formed in each consecutive cell, whereas intercellular hyphae ramify along the length of the whole colonised root. There were no consistent effects of soil P concentration on the percentage of the colonised root containing intercellular hyphae, that is, their response to soil P concentration followed that of the total colonisation.

The effects of soil P concentration on percent colonisation in this experiment are generally in accordance with published observations (Sanders & Tinker, 1971; Daft and Nicolson, 1972; Bruce *et al.*, 1994; Smith & Read, 1997; and many others). With increased soil P concentration there was a small decrease in percent colonisation between P2 and P3. The decrease in colonisation suggests that the critical soil P concentration, with respect to a reduction in total

colonisation, is between P2 and P3. The observed effect of P addition on the root length colonised of *Allium* spp. has been well documented (Smith & Gianinazzi-Pearson, 1990; Bolan, 1991; Smith & Read, 1997, and many others). A reduction in colonisation due to increased soil P can be attributed to a reduction in the formation of entry points (per unit root length) or a decrease in the growth of the fungi relative to the growth of the roots (or vica versa) (Bruce *et al.*, 1994). At the 4 and 6 week harvests, with increasing soil P concentration, there was a significant increase in the RFW, which in part explains the decrease in the percentage of the root length colonised.

Much of the work on the effects of soil P concentration on the morphological development of AM has been conducted using *Allium* spp. (Amijee *et al.*, 1989; Smith & Gianinazzi-Pearson, 1990; Pearson *et al.*, 1991; Dickson *et al.*, 1999a, b) and therefore provide a good comparison to this experiment. The reported effects of P addition on the morphological development of AM in the literature are inconsistent. For example, it has been demonstrated that with increased soil P concentration there is no effect on the production of entry points (for example, Pearson *et al.*, 1991; Bruce *et al.*, 1994). Conversely, there are reports that increased soil P concentration has been shown to result in a decrease in the formation entry points (for example, Amijee *et al.*, 1989; Smith & Gianinazzi-Pearson, 1990). In this experiment soil P concentration was found to have no significant effect on the formation of entry points per unit colonised root length (measured as EC). However, although the numbers of entry points per

length of colonised root did not decrease, the total colonisation did, therefore there was an effective decrease in the formation of entry points per unit root length. Thus, the decrease in colonisation observed here can be explained by differences in the rate of root growth (RFW) relative to that of the fungi and decreased formation of entry points per unit root length.

The results presented here demonstrate that increased soil P concentration resulted in a reduction in the percentage of the root containing arbuscules at 4 and 6 weeks. Therefore, the effect of P on arbuscule formation is not simply due to a decrease in total colonisation, but rather an effect on arbuscule production itself. As with the formation of entry points, different effects of soil P concentration on the formation of arbuscules are reported in the literature. Dickson *et al.* (1999a) found that at 4 weeks there was no effect of P addition on the root length containing arbuscules, whereas at 6 weeks there was a decrease. A number of other experiments have also shown a decrease in the formation of arbuscules with increased soil P concentration (for example; Braunberger *et al.*, 1991; Pearson *et al.*, 1991; Bruce *et al.*, 1994). Conversely, it has also been shown that at a range of soil P concentrations the development of arbuscules is not affected (for example, Smith & Gianinazzi-Pearson, 1990). Since P addition can result in a decrease in the root length containing arbuscules (Braunberger *et al.*, 1991; Bruce *et al.*, 1994), or result in decreased size of arbuscules (Mosse, 1973), these findings are important as the arbuscules are often considered to be the site of P transfer to the plant. For example, changes in the formation of arbuscules in

response to altered soil P conditions may reflect changing requirements of the plant and the fungi.

Although the results presented here support some previous experiments and not others, this is not unexpected given the inconsistencies reported in the literature. However, they do not explain the absence of arbuscules in Dr Ayling's experiment. Arbuscule production has been shown to be influenced by a number of factors for example age of AM associations and light levels (Hayman, 1974; Smith and Gianinazzi-Pearson, 1990; Pearson et al 1991; and others). Unfortunately the light levels were not measured in Dr Ayling's experiment and the age of the plants is unknown, however, these factors may in part explain the absence of arbuscules.

4.4.2 Plant growth

As with the effects of soil P concentration on the colonisation of plants by AMF, the effects of growth of colonised and uncolonised plants have been extensively studied. As a whole the results presented here agree with many of the previously published experiments. At the 2 week harvest there was no apparent growth response. The effect of P addition to soils with low P concentrations on plant growth has been well documented previously, as has the lag in growth response to P addition to the soil (Bruce *et al.*, 1994). At the 2 week harvest, the percentage of the root length colonised was low and there was little or no internal colonisation, that is, the fungi had not progressed beyond the stage of

appressorium formation and initial penetration of the roots. This, coupled with any remaining seed P reserves, may explain the lack of a growth response at the 2 week harvest. Although, at this early stage there were no measurable growth differences, there may have been increased P uptake in the colonised plants. A similar delay in benefit was observed by Dickson *et al.* (1999a) who found that growth responses were only apparent at later harvests. At the 4 week harvest the only effect of colonisation was a decrease in growth at P3. This may have been due to a large carbon drain in this treatment, as observed in other experiments (see Johnson, Graham & Smith, 1997; Graham & Eissenstat, 1998).

At the 6 week harvest there was a positive growth response, in terms of SDW, due to colonisation except at P3 where there was no significant effect. Son & Smith (1988) found that as soil P concentration increased, the response in terms of dry weight decreased, at both high and low light levels. This is similar to the results of both Smith & Gianinazzi-Pearson (1990) and Pearson *et al.* (1991) who observed a positive growth response only at low soil P concentrations. The lack of an effect at P3 may be that the balance between the costs and benefits of forming AM have "cancelled" each other out. This lack of a growth response at the highest soil P concentration is similar to that reported by Abbott & Robson (1977, 1978), Schubert & Hayman (1986) and Thomson *et al.* (1986).

The increased root weight (at 6 weeks) can be attributed to the increased soil P concentration. At the 6 week harvest the root fresh weight was significantly

increased at all levels of P addition. This suggests that the soil P concentration was below the optimum for *Al. porrum* and as a result, addition of P increased root growth, reflecting an attempt by the plant to forage for more of the P in the soil by increasing root biomass.

It has been widely reported that the colonisation of roots by AMF can result in a decrease in root biomass at constant soil P as the mycorrhizal hyphae reduce the requirement for root production by taking over, to an unknown extent, the acquisition of P from the soil (Smith & Read, 1997). A possible reason for the lack of an effect of mycorrhizal inoculation on root fresh weight may be because any mycorrhizal effects were obscured by effects of high soil P on root growth.

4.5 Conclusions

Al. porrum inoculated with *S. calospora* formed an *Arum*-type morphology with abundant arbuscules in contrast to previous results under similar conditions (S. Ayling, unpublished results). The addition of P to the soil not only influenced plant growth but also had an effect on the extent and some aspects of morphology of colonisation, consistent with many previous reports. The production of entry coils was not influenced by soil P concentration, whereas arbuscule production was. Although these results to do not support the reported absence of arbuscules, they provide useful information on the effect of soil P concentration on AM

morphology, that give a basis for comparison of effects of soil P concentration on the development of *Paris*-type AM (see Chapter 7).

CHAPTER 5 THE INTERDEPENDENCE MAGNIFIED INTERSECTS TECHNIQUE (IMIT): TIME-COURSE OF DEVELOPMENT OF *ASPHODELUS FISTULOSUS/GLOMUS CORONATUM* SYMBIOSIS

5.1 Introduction

Part of the work presented in this chapter has been published in the *New Phytologist*:

Cavagnaro TR, Smith FA, Lorimer MF, Haskard KA, Ayling SM, Smith SE. 2001. Quantitative development of *Paris*-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum*. *New Phytologist* **149:** 105-113. (Bound at rear of thesis).

Unlike *Arum*-type AM, there are few quantitative data on development of *Paris*-type AM, including the time-course of development. This is an important gap in our knowledge given the more frequent occurrence of *Paris*-type AM in nature. The aim of the work presented in this chapter was to study the development of different structures typical of *Paris*-type AM, that is, intracellular hyphal coils and arbusculate coils, to determine if the 'simple' hyphal coils represent a stage in the development of arbusculate coils.

It is well established there are three distinct phases in the formation of a colonised root system, the lag phase, a phase of rapid increase in colonisation and finally a steady state or phase of maintenance of colonisation (Sanders *et al.*, 1977). The development of these three phases is related to the relative growth rates of the roots and the AMF. At the structural level, a number of quantitative time-course studies of the development of *Arum*-type AM have been undertaken (for example, Carling & Brown, 1982; Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997). However, in some morphological studies, associations of an unknown age have been used (Carling & Brown, 1982). As a result, it has been difficult to quantify important stages in the morphological development of AM accurately with respect to time. This problem was overcome by Brundrett *et al.* (1985) who developed the nurse-pot technique, in which plants were transplanted into an actively growing pot culture of an AM fungus associated with *Al. porrum* plants that had been growing for several months. This results in near-synchronous colonisation of the plant and allows for the observation of progressive development of root colonisation by the fungi. The method was modified by Rosewarne *et al.* (1997), by using younger nurse-plants in order to reduce the risk of contamination of the pots by fungal pathogens, to increase the ease of harvesting, and to produce synchronously colonised root material suitable for time-course studies very rapidly.

The magnified intersects technique (MIT) (see section 2.4.5.2) provides a count of intersections containing different AM structures. However, the

interdependence of the different structures, that is, their co-occurrence, is not taken into account. This is particularly relevant in studies of the time-course of development of individual structures and their relationships to each other.

Specifically, the aims of the work presented in this chapter were therefore:

I To modify the magnified intersects technique to allow for the quantification of the interdependence between different AM structures, allowing for the relationships between structures over time to be determined,

II To use this new method to quantify the time-course of development and interdependence between the different structures (external hyphae, hyphal coils and arbusculate coils) formed by *Glomus coronatum* when forming *Paris*-type AM with *A. fistulosus* grown in nurse-pots,

III To assess the growth of *A. fistulosus* and its responsiveness to colonisation by *G. coronatum*.

5.2 Materials and methods

5.2.1 Plant growth & harvesting

Al. porrum nurse-pots were established as described in section 2.3.3. Forty two days after planting the *Al. porrum* nurse-plants, soil was removed from the centre of each nurse-pot using an 8 mm diameter cork borer. One carefully washed *A. fistulosus* seedling (grown for 2 weeks in 200 mm diameter, plastic, free draining pots, containing a sterile 1:9 (w/w) mixture of Mallala soil and sand) was placed into the hole and the soil removed using the cork borer was backfilled. Seedlings of a uniform size were selected for transplanting in order to minimise variation between the replicates.

The *Al. porrum* (nurse-plants) and *A. fistulosus* plants were harvested as described in section 2.4.1. Shoot material was used for the determination of dry weights. Root material was cleared and stained for assessment of colonisation by the fungi (see section 2.4.4). Four replicate pots were harvested each day for the first 21 days after transplanting (DAT) and then 23, 25 and 27 DAT. Four replicate non-mycorrhizal control pots were harvested 4, 8, 12, 16, 20, 24 and 28 DAT.

5.2.2 Determination of spatial separation of coil types

The aim was to determine the location of hyphal coils (HC) and arbusculate coils (AC) in different root cortical cell layers. The cortex of *A. fistulosus* is made up of four cell layers, accordingly, the inner and outer cortex were defined as the two cell layers immediately adjacent to the stele and epidermis respectively. At 10, 15, 20 and 25 DAT stained root material from the two replicate slides from each of the four replicate plants was evaluated for the location of HC and AC. For plants harvested 10 DAT, data were based on between six and nine intersections per plant, due to low overall colonisation at that time. AC were not observed in these samples and intersections with colonisation in both the inner and outer cortex were scored. In samples from 15, 20 and 25 DAT, intersections (20 per slide) containing HC and AC together were chosen and the location in the cortex of the two types of coils (HC and AC) was scored. The means and standard errors were calculated for each harvest time.

5.2.3 A new method for the assessment of colonisation: the Interdependence, Magnified Intersects Technique (IMIT)

The colonisation of roots was scored using a modification of the method of McGonigle *et al.* (1990) as described in section 2.4.5.2. At each intersection the presence of AM structures (external hyphae (EH), HC and AC and intersections

containing no fungal structures) was scored. Until this point all of the methods used to score the colonisation were the same as the original MIT.

Rather than simply recording the total counts of intersections of each type of AM structure for each slide, the presence/absence of each of the structures at each intersect from each slide was recorded separately. This gave a total of 50 observations for each slide and 100 for each plant. The information from all observations from each slide was also summed to give the same information as from using the original MIT.

Examples of the method for recording data using the original MIT and the new IMIT are given in Tables 5.1 and 5.2 a & b, respectively. Note that the same information using the MIT is gained using the IMIT. However, the IMIT records which structures occur together and which do not. The examples given here (Table 5.2 a, b), illustrate that the same totals are recorded, but that there is a very different relationship between the structures.

Table 5.1 Typical data collected using the MIT. Zeros represent sections where structures were not observed.

	EH	**HC**	**AC**	**Zeros**	**Total Intersections scored**
Category totals	**4**	**4**	**4**	**4**	**10**

Table 5.2 Data collected using the interdependence IMIT, (a) illustrating a strong relationship between EH and HC and (b) different relationships between the structures compared to 5.2 a. Note that totals are the same as in Table 5.1. Zeros represent sections where no structures were observed.

(a)

	EH	HC	AC	Zeros	Total Intersections scored
	1	1			1
	1	1			2
	1	1	1		3
	1	1	1		4
			1		5
			1		6
				1	7
				1	8
				1	9
				1	10
Category totals	**4**	**4**	**4**	**4**	**10**

(b)

	EH	HC	AC	Zeros	Total Intersections scored
			1		1
		1	1		2
	1	1	1		3
	1	1	1		4
	1	1			5
	1				6
				1	7
				1	8
				1	9
				1	10
Category totals	**4**	**4**	**4**	**4**	**10**

5.2.4 Modelling of Interdependence Magnified Intersects Technique data

The data for each Harvest, Plant and Slide (2 per plant) were summarised in a 2x2x2 contingency table corresponding to the number of independent intersections (out of 50) with EH (+ or -), HC (+ or -), and AC (+ or -). The

contingency table for 18 DAT, Plant 4 Slide 1 is presented as an example (Table 5.3). The response variable is the number of intersections with each combination of EH, HC and AC (at each Harvest), therefore, a log-linear model assuming a Poisson distribution was considered to be appropriate for these data. All statistical analysis was performed using S-PLUS (Mathsoft Inc, USA), by Ms Michelle Lorimer and Ms Kathy Haskard, BiometricsSA. The response variable was transformed with the log link transformation in order to ensure that the mean was always positive.

Table 5.3 2x2x2 contingency table for 18 DAT, Plant 4 Slide 1. Showing the numbers of EH, HC and AC in their various combinations.

AC=No

EH \ HC	No	Yes	
No	28	2	30
Yes	8	0	8
	36	2	38

AC=Yes

EH \ HC	No	Yes	
No	1	4	5
Yes	2	5	7
	3	9	12

When all categorical factors were included, the data were considered to form a 6 way contingency table, made up of the categorical factors, Harvest, Plant, Slide, EH, HC and AC. For these data, EH, HC and AC were the response factors and Harvest was the stimulus factor of interest. Of main interest is the relationship between the multinomial response and the stimulus factor, Harvest. Therefore, in the generalised linear model those interactions between Harvest and combinations of EH, HC and AC were considered. Plant and Slide are "nuisance" factors, but were included to ensure that the marginal totals are fixed equal to their observed values. Thus, the maximal model has the following form:-

$$\left.\begin{aligned} log(\mu)_{ijkrst} = \lambda + (Harvest)_i + (Plant)_j + (Harvest.Plant)_{ij} + (Harvest.Plant.Slide)_{ijk} \\ + (EH)_r + (HC)_s + (AC)_t + (EH.HC)_{rs} + (EH.AC)_{rt} + (HC.AC)_{st} + (EH.HC.AC)_{rst} \end{aligned}\right\} \text{A}$$

$$\left.\begin{aligned} + (Harvest.EH)_{ir} + (Harvest.HC)_{is} + (Harvest.AC)_{it} + (Harvest.EH.HC)_{irs} \\ + (Harvest.EH.AC)_{irt} + (Harvest.HC.AC)_{ist} + (Harvest.EH.HC.AC)_{irst} \end{aligned}\right\} \text{B}$$

Where:-

A = Minimal model, B = Interactions between the response and stimulus variables of interest. μ_{ijkrst} is the expected mean number of intersections for the *k*th Slide of Plant *j* harvested at time *i* with *EH* = *r*, *HC* = *s*, and *AC* = *t*. $(Harvest)_i$ is a parameter for the *i*th Harvest time; $(EH)_r$ is a parameter for the *r*th level of EH, and so on; $i = 1,...,24$; $j = 1,2,3,4$; $k = 1,2$; r = 0,1; s = 0,1; t = 0,1. λ is an intercept parameter.

Terms were progressively dropped from the model and their importance was determined by assessing the change in the residual deviance. The non-significant terms were removed from the full model. The remaining (significant) terms demonstrate the relationships between Harvest and the structures scored. The residual deviance is analogous to the residual sum of squares in analysis of variance, in that it is a measure of the lack of congruence between the observed and fitted values. The final fitted model provides estimates of the expected numbers in each of the combinations of EH, HC and AC at each Harvest.

5.3 Results

5.3.1 Plant growth

The SDW of inoculated and uninoculated plants are presented in Figure 5.1. There was little difference in the growth of the plants whether they were inoculated with *G. coronatum* or not. Furthermore, after transplanting there was little increase in the SDW of the plants for either treatment over the course of the experiment.

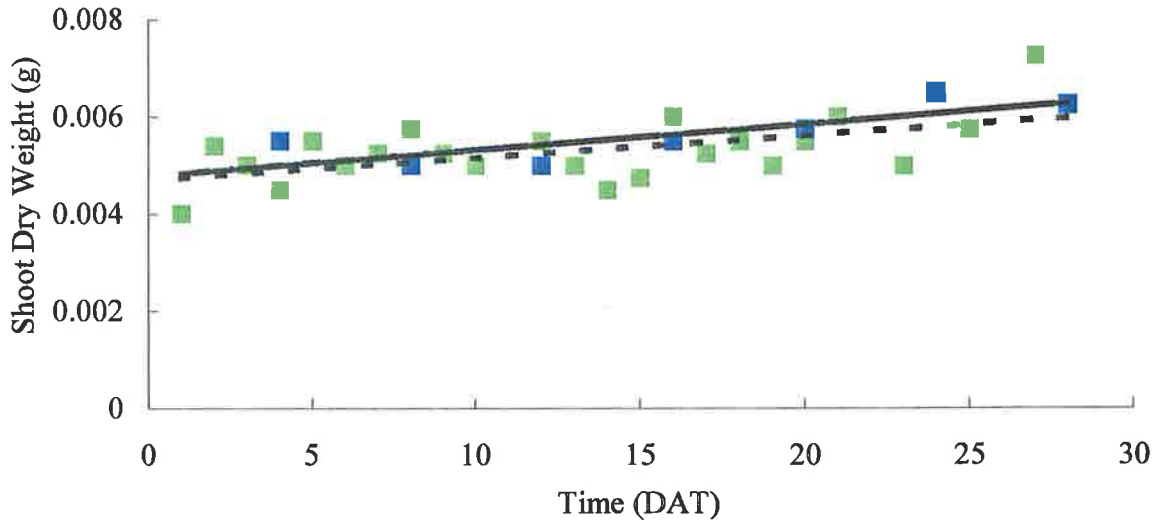


Figure 5.1 Mean shoot dry weights of *A. fistulosus* inoculated with *G. coronatum* (■) and uninoculated controls (■) plants after transplanting. Linear regression lines were fitted to the data of the inoculated (solid line) and uninoculated (dashed line) plants, the R^2 values were 0.65 and 0.3 respectively. (n=4).

5.3.2 Morphology and location of hyphal coils and arbusculate coils in the inner and outer cortex

The colonisation process began with hyphae in the soil becoming attached to the surface of the roots, forming the first EH. This was followed by penetration of the epidermal cell layers of the roots, leading to the production of intracellular HC first in the outer and then in the inner cortex. Finally, AC were formed intracellularly, predominantly in the inner cortex. At no stage was there an intercellular phase of colonisation.

HC were composed of loops of thick hyphae, 5 to 8 μm in diameter. The only apparent difference between the AC and HC was that the main coiled hyphae of the AC had small, highly branched, fine arbuscule-like structures arising from them. The AC were similar to the arbusculate coils described by Yawney & Schultz (1990). Examples of HC and AC are shown in Figures 5.2 a & b.

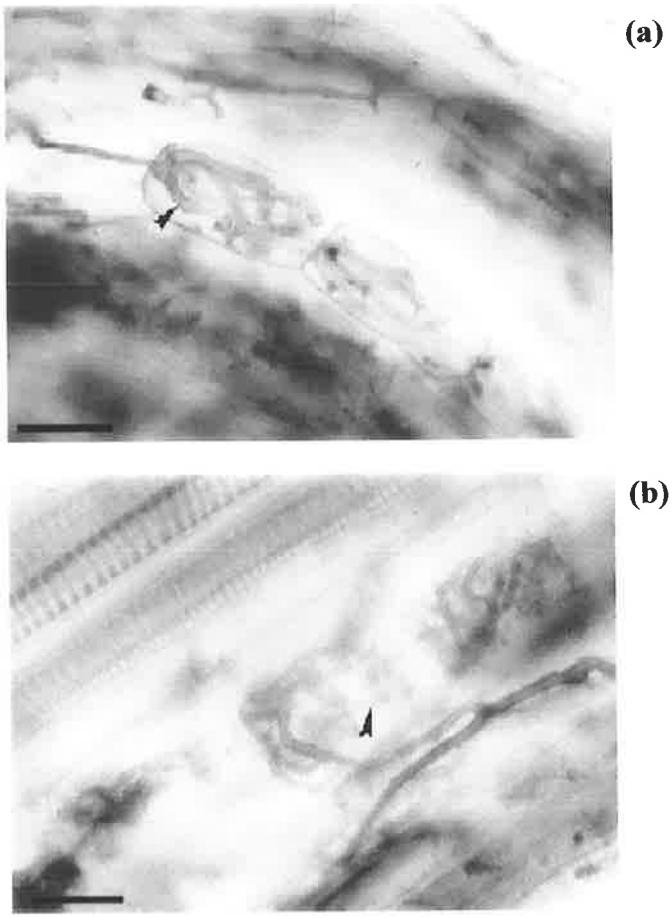


Figure 5.2 Colonisation of *A. fistulosus* by G. *coronatum*. (a), Hyphal coils, indicated by arrow, (b), Arbusculate coils, indicated by arrow. Bars = 50 µm.

Data giving the location of HC and AC in roots harvested 10, 15, 20 and 25 DAT are presented in Table 5.4. Ten DAT AC were observed in only one intersection. HC were always found in the outer cortex (100%) and were found in the inner cortex in approximately half (56.8%) of the intersections viewed. At later times (15, 20 and 25 DAT) it is clear that in regions of the root where AC and HC were present together, HC were always found in the outer cortex (100%) and occasionally also in the inner cortex (3.1 - 8.1%). In the same colonised regions of the roots, AC were rarely found in the outer cortex (6.3 - 8.8%) and almost always the inner cortex (98.8 - 100%).

Table 5.4 Percent occurrence of hyphal coils and arbusculate coils in the inner and outer cortex of roots at 10, 15, 20 and 25 DAT. Values are means ±SE. n=20 except for 10 DAT where n=6-9.

DAT	HC %		AC %	
	Outer	**Inner**	**Outer**	**Inner**
10	100 ± 0	56.8 ± 8.5	0 ± 0	4.2 ± 4.2
15	100 ± 0	8.1 ± 2.5	8.8 ± 2.3	98.8 ± 1.3
20	100 ± 0	7.5 ± 2.3	8.1 ± 1.9	100 ± 0
25	100 ± 0	3.1 ± 1.3	6.3 ± 1.3	100 ± 0

5.3.3 Time-course of development

The mean percentage of the roots colonised by the fungi is given in Figure 5.3 a. Colonisation started 3 DAT the *A. fistulosus* seedlings into the nurse-pots. The percentage of the roots colonised slowly increased until 6 DAT, after which there was a steady increase in colonisation which continued to the end of the experiment. The colonisation by EH, HC and AC all followed the same pattern as percent colonisation, as seen in Figure 5.3 b, c and d. The first EH, HC and AC were observed 3, 5 and 11 DAT respectively.

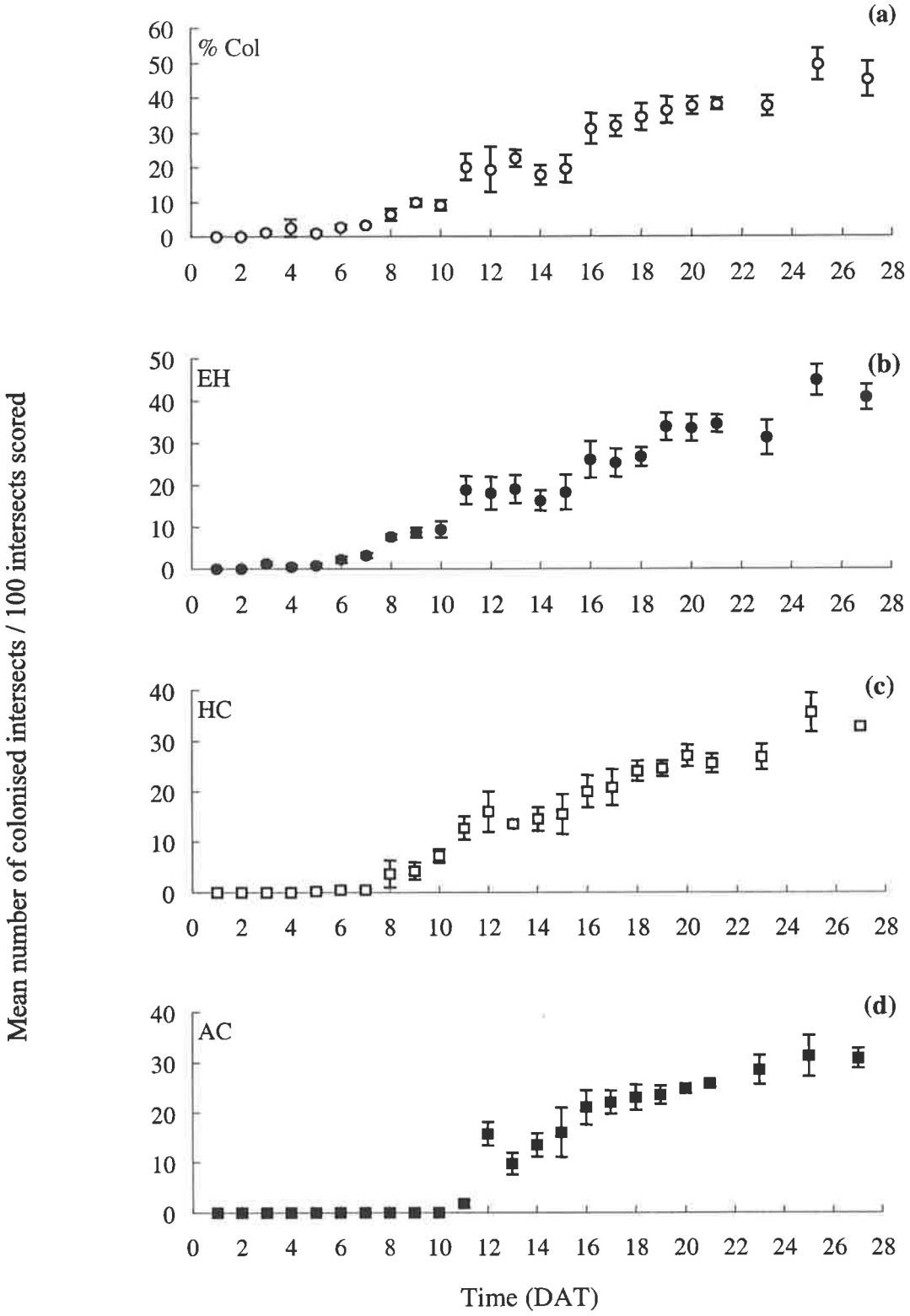


Figure 5.3 Colonisation of *A. fistulosus* by *G. coronatum.* (*a*), Root colonisation (% Col.), (*b*), EH, (*c*), HC, (*d*), AC. Bars are standard errors. (n=4).

5.3.4 Selection of model for assessing interdependence over time

The data collected using the IMIT were modeled to select a final model, which explained the interdependence of the different structures over time. Before terms were dropped from the full model, the residual deviance obtained was 1026.14 on 1162 degrees of freedom. The dispersion parameter (the ratio of the residual deviance to the degrees of freedom) was 0.88, indicating no overdispersion (McCullagh & Nelder, 1995).

The four-way interaction between Harvest, EH, HC and AC was not significant at the 5% level, and so this term, $(Harvest.EH.HC.AC)_{irst}$, was permanently removed from the model. Modelling continued by the removal of three-way interaction terms involving Harvest until a model in which all of the remaining three-way interaction terms were significant at the 5% level. In this model, AC did not appear in any of the three-way interactions. However, removal of the interaction between AC and Harvest showed that there was a very strong interaction between them. The final model is given below:-

$log(\mu_{ijkrst})$= Minimal model + $(Harvest.EH)_{ir}$ + $(Harvest.HC)_{is}$ + $(Harvest.EH.HC)_{irs}$ + $(Harvest.AC)_{it}$

There was a significant three-way interaction between Harvest, EH and HC, and a significant two-way interaction between Harvest and AC. For ease of

presentation of the results, in all figures the expected number of intersections (out of 400) containing the associated structures, and hence corresponding percentages, have been calculated and are presented below.

5.3.5 Interdependence

The model demonstrated that there was a significant interaction between Harvest and AC and that there was a significant interaction between Harvest and the combination of EH and HC. The Harvest.AC interaction is illustrated in Figure 5.4. This shows the fitted percentages of intersections with AC, out of the intersections with each of the four combinations of EH and HC. For example, at 21 DAT, of the intersections containing both EH and HC, 89% had AC and 11% did not have AC. The percentage of intersections containing AC, out of the intersections with a given combination of EH and HC, is greatest when there are both EH and HC together, next greatest when only HC were present, lower when only EH were present, and lowest (negligible) when EH or HC were present.

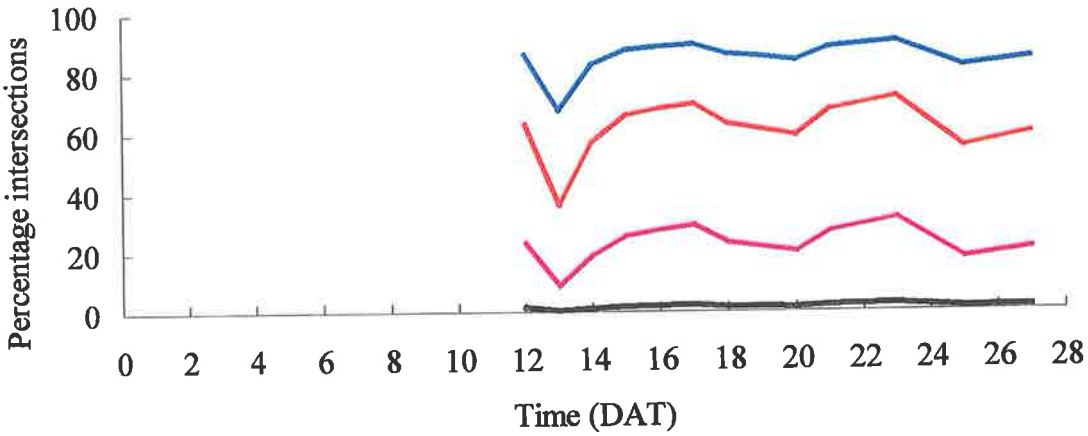


Figure 5.4 Percentage of intersections containing AC, given the combination of EH and HC over time. Both EH and HC (—), HC only (—), EH only (—) and neither EH or HC (—). (n=4).

The Harvest.EH.HC term in the model is illustrated by the results shown in Figures 5.5 a & b. These graphs show the percentages of intersections with each combination of EH and HC when AC was absent (Figure 5.5 a) or present (Figure 5.5 b) across Harvests. No AC were present before 11 DAT and there were only 7 intersections containing AC out of a total of 400 at 11 DAT. Therefore, results relating to intersections with AC present are shown from 12 DAT onwards. When AC is absent (Figure 5.5 a), there are many more intersections containing neither EH or HC than the other combinations of EH and HC. Conversely, as seen in Figure 5.5 b, when AC was present there were many more intersections containing both EH and HC together compared to the other combinations of EH and HC. Together Figures 5.4, 5.5 a and 5.5 b demonstrate that it is more likely that all three structures will be formed together or all three structures will be absent than the other possible combinations of EH, HC and AC.

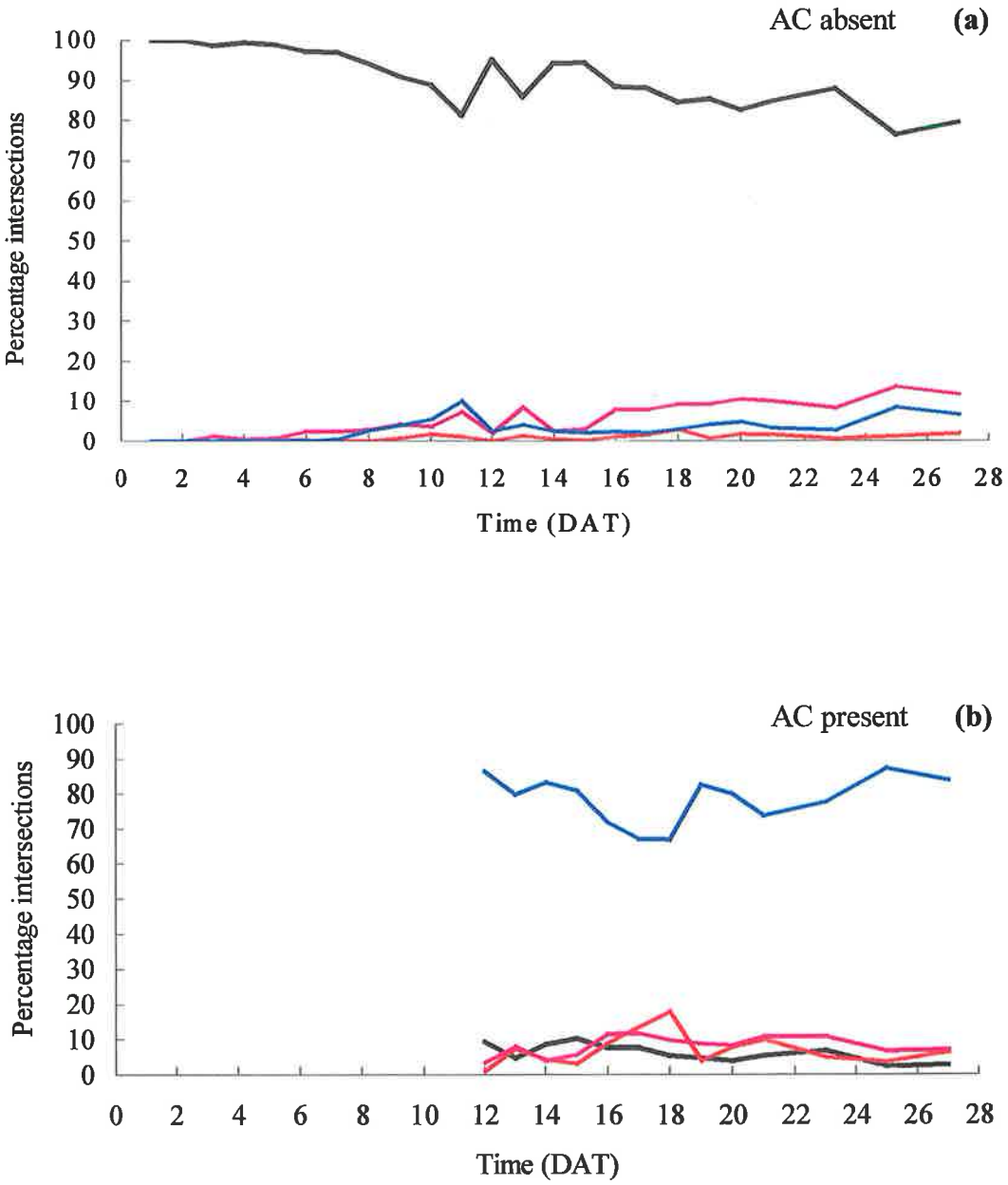


Figure 5.5 Percentages of intersections with either, both or neither EH and HC when (a) AC is absent or (b) AC is present at each time. Lines on the graph represent Both EH and HC (—), HC only (—), EH only (—) and neither EH or HC (—). (n=4).

Comparison of the fitted values from the final model in which the dependence between EH and HC changed over time, and the expected values assuming independence of EH and HC demonstrated that at each Harvest, EH and HC were observed together more often, and were both absent more often, than would be expected if they were independent. The ratio of the predicted counts of EH and HC together for the fitted model and the expected counts of EH and HC if these structures developed independently are given in Figure 5.6. If the structures were independent, a ratio of unity would be expected. For example, at 27 DAT the model predicts 122 intersections with EH and HC together, and 224 with neither. If EH and HC were independent approximately 55 would have EH and HC together and 157 would have neither, from totals of 163 with EH and 135 with HC out of 400. Therefore, 27 DAT, the ratio of predicted counts to expected counts is 122/55 (ratio = 2.18). Thus, there were more intersections with EH and HC together than expected if they were independent of each other. The same trend was apparent for all Harvests where EH and HC were present.

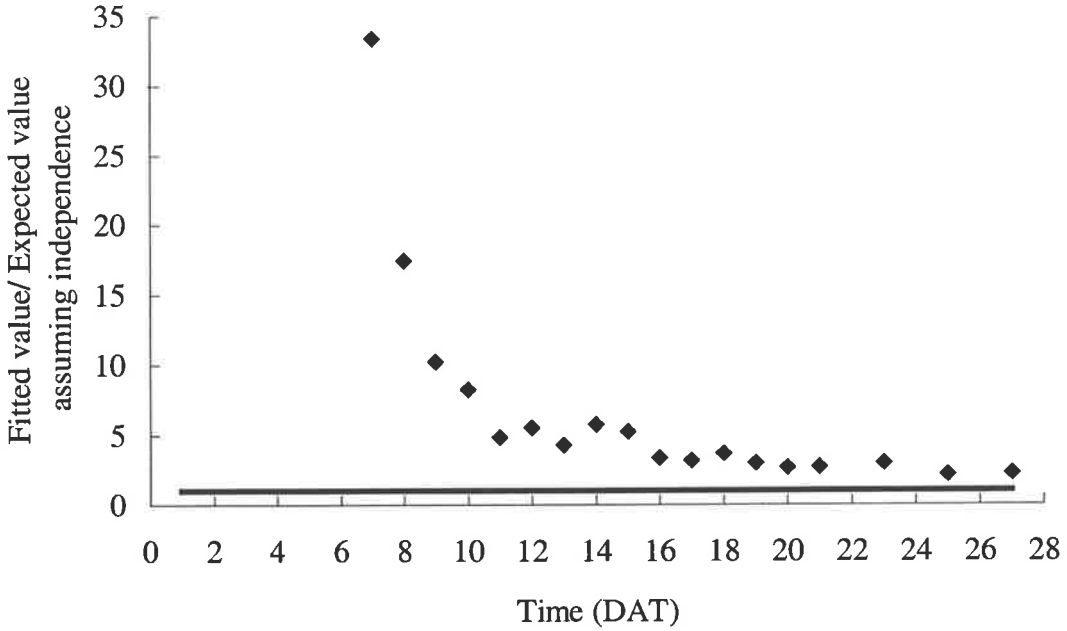


Figure 5.6 Level of dependence of EH and HC over time. Ratio of fitted values under the final model to expected values from a model assuming independence. The horizontal line indicates the expected ratio of unity (=1) if independence holds. (n=4).

5.4 Discussion

5.4.1 Plant growth

Prior to transplanting, the *A. fistulosus* seedlings grew steadily with time (data not shown). After transplanting there was a very small increase in the SDW of both the mycorrhizal and non-mycorrhizal plants, and the growth of the colonised plants did not differ significantly from those of the non-colonised plants (see Figure 5.1). This is quite different from the results reported by Rosewarne *et al.* (1997) with *Ly. esculentum* grown in nurse-pots with *Al. porrum*, where the colonised plants were larger than the uncolonised plants. The poor growth of the *A. fistulosus* plants after transplanting may be because the *Al. porrum* plants had exhausted the available P in the soil, so that the very small *A. fistulosus* plants were unable to compete effectively even when they had become mycorrhizal. Alternatively, the observed lack of a response to colonisation by *A. fistulosus* may be simply because *A. fistulosus* is not responsive to colonisation, at least under the conditions in this experiment. Therefore, it was decided to investigate the growth and P nutrition of colonised and uncolonised *A. fistulosus* plants (see Chapter 7).

5.4.2 Morphology and location of HC and AC in the inner and outer cortex

The HC and AC formed in *A. fistulosus* by *G. coronatum* are typical *Paris*-type structures, similar to those observed in *Ac. saccharum* (Yawney & Schultz, 1990; Cooke *et al.*, 1993). As found previously (Gallaud, 1905; Whitbread *et al.*, 1996), some HC are a developmental step leading to the formation of AC. The results of this experiment demonstrate that in the *A. fistulosus/G. coronatum* symbiosis the vast majority of HC in the inner cortex develop into AC. However, HC in the outer cortex do not develop into AC. The two types of coils are therefore distinct.

The spatial separation of HC and AC in different regions of the cortex has not previously been demonstrated quantitatively. If all HC developed into AC, we would expect to see a reduced rate of HC production as the production of AC increased. This was not the case. Conversely, if HC and AC occur in different parts of the same section of root, we would not necessarily see a dramatic change in the rate of production of HC and AC, as was the case (Figures 5.2 c and d). Therefore, in *A. fistulosus* colonised by *G. coronatum* HC in the outer cortex are distinctly different structures from those in the inner cortex in that the majority of the latter become AC. The development of HC in the inner cortex into AC may be controlled by differences in the structure, physiological signals and function of the cortical cells. Interestingly, McGonigle *et al.* (1999) rarely saw HC and AC in

the same intersections in *Pa. quinquefolius* roots, that is, the spatial separation observed here was not observed in their samples.

A pattern of arbuscule production in cells of the inner cortex around the stele occurs in many (but not all) *Arum*-type AM, for example, *T. subterraneum* (*Arum*-type) inoculated with a range of *Glomus* species Abbott (1982). Blee & Anderson (1998) also noted that arbuscules were only formed in cortical cells immediately adjacent to the endodermis in *Zea mays*, *Triticum aestivum*, *Daucus carota*, *T. subterraneum*, *Vigna radiata* and *Phaseoleus vulgaris*. Similar spatial differences in the production of HC and arbuscules have also been observed in the *Paris*-types *Li. tulipifera* (Kinden & Brown, 1975a) and *Pa. quinquefolius* (Whitbread *et al.*, 1996). However, the coils in *Pa. quinquefolius* were not predominantly restricted to the outer cortex as observed in *A. fistulosus* and in *Li. tulipifera*. The transition of HC to AC must be a rapid process and related to location within the root. This is important, because it suggests that the differences in the morphology are influenced by both time and the stage of differentiation of the root.

5.4.3 Time-course of development

As shown in Figure 5.3 a, the percent colonisation followed a pattern similar to that observed in previous time-course studies of root colonisation in *Arum*-type AM. There was an initial lag followed by an increase in rate of colonisation, as

described by Sanders *et al.* (1977) for several fungi forming AM with *Al. porrum.* By the end of the experiment (27 DAT) the rate of increase of percent colonisation in *A. fistulosus* had slowed only slightly, suggesting that the steady state of colonisation or phase of maintenance of colonisation (Sanders *et al.*, 1977) had not yet been reached. However, in a similar nurse-pot study of *Ly. esculentum* colonised by *G. intraradices* (*Arum*-type), a plateau of approximately 95% colonisation was reached within 16 DAT (Rosewarne *et al.*, 1997). If the current experiment had been conducted over a longer period of time it is anticipated that this plateau would have been reached. This is supported by another experiment with *A. fistulosus* and *G. coronatum* where the colonisation reached a maximum of 60% after 42 days (Chapter 7).

In general the fractional EH, HC and AC colonisation of the roots all followed the same pattern as the percent colonisation. The presence of EH was first noted 6 DAT (Figure 5.3 b). This is later than previous reports for *Ly. esculentum* and *Al. porrum* (both *Arum*-type plants) in which 25% and 15% of intersects had EH at 2 DAT respectively (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997). Furthermore, the maximum level of EH colonisation was reached within 8 DAT (Rosewarne *et al.*, 1997), but in *A. fistulosus* EH continued to increase throughout the 28 days of the experiment and only reached 49%.

The results of the experiment presented here are in agreement with field studies of *Paris*-type colonisation which indicate that the process may be slow,

compared with *Arum*-type colonisation (Brundrett & Kendrick, 1988; McGonigle *et al.*, 1999). The fast growth rate of hyphae in the intercellular spaces of *Arum*-type roots may be due to lower resistance compared with the intracellular path in *Paris*-type roots (Brundrett *et al.*, 1985). Due to the tortuous nature of the hyphae forming the coils in *Paris*-type AM, the same length of hyphae may result in a lower length of colonisation unit compared with *Arum*-type AM.

5.4.4 The Interdependence Magnified Intersects Technique

As stated by McGonigle *et al.* (1990) there are three main methods for the visual quantification of root colonisation: (1) subjective estimation, (2) calculation of the percentage of root segments or microscope fields of view that may contain any colonisation, and (3) grid line intersect methods. Although the MIT gives the fractional colonisation of the different fungal structures (McGonigle *et al.*, 1990), it does not allow for the determination of the interdependence of the different structures, where the IMIT does.

The IMIT for scoring colonisation described here allowed for the determination of the interdependence of the different *Paris*-type structures over time. The IMIT is not limited to evaluation of mycorrhizal colonisation. In any study where the aim is to determine the interdependence of any two or more structures or signals (for example; physiological stains or molecular probes or antibodies) the IMIT can be applied by scoring the data and analysing it in the

manner described above. Since publication of the IMIT (Cavagnaro *et al.*, 2001c) it has been used to relate metabolic activity of arbuscules to the presence and absence of cross wall in *Arum*-type AM (Dickson & Smith, *in press*).

The final model revealed a number of interactions that were significant. It is possible to interpret the significant interactions by looking at the expected mean number, or corresponding percentages, of intersections with the associated structures. Figure 5.4 shows that of intersections with both EH and HC, most (approximately 80%) will contain AC (after 11 DAT). In situations where HC are present and EH absent, the proportion of AC is next highest (approximately 60%), followed by EH only present (approximately 25%) and finally neither EH or HC (negligible). The fact that the four lines in Figure 5.4 are approximately parallel illustrates that although there is a significant interaction between Harvest and AC, the combination of EH and HC does not influence this interaction. This demonstrates that although the *pattern* over time of the proportions of intersections with AC is the same regardless of the combination of EH and HC, though the *actual* proportions are different for each combination of EH and HC. That is, the relationships between the structures over time are the same, even though the number of structures may change.

Figures 5.5 a & b give the relative proportions of intersections containing the four combinations of EH and HC across time in the absence (Figure 5.5 a) and presence (Figure 5.5 b) of AC. The most striking feature is that in the presence of

AC, EH and HC together was the most likely combination. Conversely, in the absence of AC other fungal structures were rarely found. Once again this demonstrates the strong relationship between fungal structures in AM. Pairs of lines (one from each of Figures 5.5 a & b) corresponding to the same combination of EH and HC are parallel, in a similar way to the parallelism in Figure 5.4. In fact, the numbers of any given combination of EH and HC relative to the numbers with another combination follow the same pattern over time, regardless of whether AC is present or absent, but the actual relative numbers do not depend on AC.

The relationships in Figures 5.4, 5.5 a & b can be explained as follows. Early in the colonisation process only EH will be found (as also seen in Figure 5.2 b). Later HC will appear (Figure 5.2 c) initially with EH, but as the HC ramify along the root HC may be found without EH. This can also be seen in the reduced dependence of EH and HC on each other over the course of the experiment (Figure 5.6). Alternatively, AC may form from HC in the inner cortex giving all three structures together, as occurs commonly (Figures 5.4 and 5.5 b). Where HC and AC are found together without EH, this represents situations where the internal colonisation has ramified beyond the entry points which are obviously associated with EH. AC tend to be found in the inner cortex associated with HC in the outer cortex (Table 5.4); therefore, the occurrence of AC without HC (with or without EH) would be rare.

5.5 Conclusions

The morphology of the AM resulting from the colonisation of *A. fistulosus* by *G. coronatum* is of the *Paris*-type confirming results presented in Chapter 3. It is apparent that the time-course of development of this *Paris*-type AM is slower than that of *Arum*-type AM previously investigated in equivalent experimental nurse-pot systems, and this may be significant in physiological studies of AM. It would be useful to test this apparent difference further by studying a wider range of plant and fungal species and environmental conditions.

Although the roots were colonised, there was no measurable increase in the shoot dry weight. The lack of growth response may be either an artifact of the experimental set-up or simply that the *A. fistulosus/G. coronatum* association is in fact not beneficial to plant growth in terms of SDW. This requires further investigation (Chapter 7).

This experiment showed that both time and space influenced the formation of the two types of coils and that the relationships between Harvest, EH, HC and AC are complex. The statistical modelling approach shows that the interactions between EH, HC and AC are strong. If AC are present, it is most likely that EH and HC will also be present. Similarly, if AC is absent, EH and HC are also likely to be absent or only present in very low numbers.

CHAPTER 6 LASER SCANNING CONFOCAL MICROSCOPY OF *ASPHODELUS FISTULOSUS/GLOMUS CORONATUM* SYMBIOSIS: THREE-DIMENSIONAL ANALYSIS OF PLANT NUCLEAR SHIFT

6.1 Introduction

Part of the work presented in this chapter has been published in the journal *Symbiosis*:

Cavagnaro TR, Kolesik P, Smith FA, Ayling SM, Smith SE. (2001). Arbuscular mycorrhizas formed by *Asphodelus fistulosus* and *Glomus coronatum*: Three-dimensional analysis of plant nuclear shift using laser scanning confocal microscopy. *Symbiosis* **30 (2-3):** 109-122. (Bound at rear of thesis).

In Chapter 5, the first detailed study of morphological development of the *A. fistulosus*/G. *coronatum* symbiosis was presented. Clear differences in the timing of morphological events, compared to *Arum*-type AM, were found. However, it is not known if hyphal coils or arbusculate coils (*Paris*-type structures) cause structural rearrangements within plant cells in a similar way to arbuscules (*Arum*-type structures).

As discussed in Chapter 2, it has been known for many years that in cortical cells containing arbuscules (*Arum*-type AM) there is an increase in the size of the plant nucleus (hypertrophy) (Gallaud, 1905; Berta *et al.*, 1990). It becomes lobed, the chromatin is decondensed (Balestrini *et al.*, 1992), and there is a delay in nuclear senescence (Lingua *et al.*, 1999). There is also a positional change of the plant nucleus from the periphery towards the centre of each cell containing an arbuscule (Balestrini *et al.*, 1992). Conversely, in the hypodermal cells of *Al. porrum* containing entry coils, Balestrini *et al.* (1992) observed no change in the position of the nuclei. Kinden & Brown (1975c) observed that in cells of *Li. tulipifera* containing arbusculate coils (which they referred to as arbuscules), that the plant nucleus was hypertrophied and located centrally within the plant cell. Aside from the observations of Kinden & Brown (1975c), there have been no measurements of the influence of *Paris*-type structures, on the size and position of plant nucleus in colonised cells.

Investigations of effects of colonisation on plant nuclei have used methods such as transmission electron microcopy (TEM) for morphological observations and positional measurements, and flow cytometry and static microfluorimetry for determination of chromatin decondensation (Berta *et al.*, 1990; Balestrini *et al.*, 1992; Lingua *et al.*, 1999). The Laser Scanning Confocal Microscope (LSCM) is a very powerful tool which has been used to study the structure, physiology and fungus/plant interactions in AM systems (Ayling *et al.*, 1997; Genre & Bonfante, 1998; Melville *et al.*, 1998; Dickson & Kolesik, 1999). The LSCM has many

advantages over conventional light and electron microscopy. These include, imaging of material stained with multiple flurochromes, collecting clear images with a greater depth of field, reduced flare and high resolution. One of the biggest advantages is the ease with which serial optical sections can be collected, digitised and used for 3-D reconstruction. The LSCM can be used to measure positional changes of plant nuclei of colonised cells in three-dimensions, where only two-dimensional measurements using TEM have been made in the past. The LSCM therefore has the potential to be used to gain further insight into modifications at the cellular level of colonised plants.

Specifically, the aims of the work presented in this chapter were therefore:

I. To determine whether the size or position of the plant nucleus in cells of *A. fistulosus* is altered by the formation of *Paris*-type AM with *G. coronatum*, using LSCM in a time-course experiment,

II. To use LSCM to collect and analyse images of the hyphal coils and arbusculate coils formed by *G. coronatum* in *A. fistulosus* for detailed morphological observations.

6.2 Materials and methods

6.2.1 Plant growth

Al. porrum/G. coronatum nurse-pots were established and *A. fistulosus* seedlings were transplanted into them using the same methods as described in Chapter 5. A total of 20 pots, 10 inoculated and 10 uninoculated, were established. Two *A. fistulosus* plants were harvested at 4, 8, 12, 20 and 28 DAT from both inoculated and uninoculated treatments. Roots were sectioned (section 2.4.6) and stained overnight with acid fuchsin (1 mg ml^{-1}) at room temperature (to stain plant and fungal cell walls) and then counter-stained with DAPI (4',6-diamindo-2-phenylindole) (1 μg ml^{-1} PBS) for 10 min at room temperature (to stain nucleic acid). Sections were then mounted on microscope slides in glycerol. A sub-sample of the roots was stained using trypan blue (section 2.4.4) and percent colonisation was determined using the grid line intersect method (section 2.4.5.1).

6.2.2 Laser Scanning Confocal Microscopy

A MRC-1000UV Laser Scanning Confocal Microscope System (Bio-Rad) in combination with Ar laser and a Nikon Diaphot 300 inverted microscope in fluorescence mode were used to visualise acid fuchsin staining (excitation at 488/10 nm and emission at 522/32 nm) and DAPI staining (351/8 nm and 455/30 nm). Samples were observed with a x 40 water-immersion objective lens with a

numerical aperture of 1·15 and working distance of 210 μm. A series of optical xy-sections, each with a 2 μm interval on the z-axis, was collected for both inoculated and uninoculated plants. Each image was averaged over 4 scans using a Kalman filtering process and saved as a digital file with the size of 768 x 512 pixels and the intensity of staining expressed in 256 levels of grey (0 = black, 255 = white).

Distortion of images due to differences in the refractive index of the immersion medium (water) of the objective lens and the combination of the embedding medium (glycerol) and the fungal and plant tissue was corrected by using the correction factor calculated by Dickson & Kolesik (1999). The axial distortion reduced the nominal z-interval between the xy-slices from 2 μm to 1.42 μm.

6.2.3 Measurements

The distance from the centre of the plant nucleus to the closest cell wall was measured in ten cortical cells containing arbusculate coils (AC) (located in the inner cortex, see Chapter 5) and ten inner cortical cells of the control plants, in sections collected from two plants at each harvest time, using Comos image analysis software (Bio-Rad, UK). The distance in the z-axis was measured by counting the number of optical slices from the centre of the nucleus to the top and

bottom of the cell and multiplied by the z-step (1.42 μm). The distance from the centre of the nucleus to the closest cell wall in the x- and y-axes was measured using a line draw function of the Comos software. Due to the brittle nature of *A. fistulosus* roots, the roots were damaged during sectioning. As a result there were not enough cells containing hyphal coils (HC) (which are located in the outer cortical cells, see Chapter 5) to make sufficient measurements of positional changes for satisfactory statistical analysis. However, in the limited sections containing HC, it was possible to make some qualitative observations of the position of the plant nucleus. The volume of colonised (cells containing AC) and uncolonised cells was measured at 8 DAT, to determine if there was a change in the volume due to colonisation.

The volume of each nucleus was determined stereologically by summing the product of the cross-sectional area of each optical slice of the nucleus by the z-step. Measurements were only made on cells where the cross sectional area could be measured without interference from fungal structures. Six nuclei were measured for each treatment from each of two replicate plants 28 DAT.

Because of the irregular shape of the nucleus (see Figures 6.1 a, 6.2 a, b, c), a stereological method to calculate nuclear volume after comparison of a number of different methods, was used. A schematic diagram of a plant cell nucleus is given in Figure 6.1 a, a sphere can be formed either around the short axis (Figure 6.1 b) or the long axis (Figure 6.1 c) and the volume calculated using

the equation $4/3\pi r^3$. If it is assumed that the nucleus forms a spheroid, the volume can be calculated using the equation $4/3\pi ab^2$ if the spheroid is rotated around the long axis (prolate spheroid) (Figure 6.1 d) or $4/3\pi a^2 b$ if it is rotated around the short axis (oblate spheroid) (Figure 6.1 e). Volumes determined stereologically and assuming the different shapes and axes of rotation (Figure 6.1 b to e), of a nucleus randomly selected from the data set, were calculated to compare results obtained by different methods.

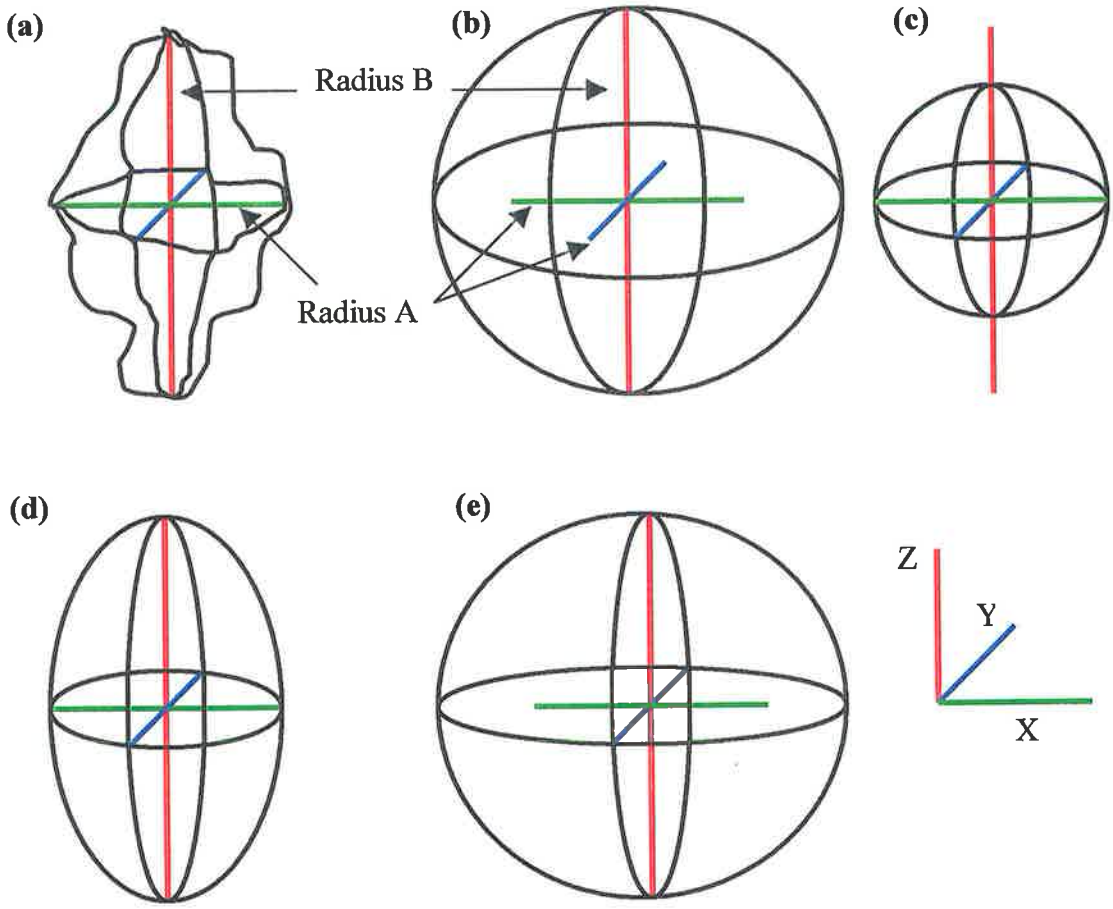


Figure 6.1 Schematic diagrams of some of the different possible shapes of a plant nucleus, based on the axis of rotation. (a) Irregularly shaped nucleus formed around its long and short axes, (b) large sphere, rotation around long axis, (c) small sphere, rotation around short axis, (d) oblate spheroid, rotation around long axis, (e) prolate spheroid, rotation around short axis.

6.3. Results

6.3.1 Development of the symbiosis

A. fistulosus colonised by *G. coronatum* formed a *Paris*-type AM with HC and AC in the inner and outer cortex of the roots respectively (as shown in Chapters 3 and 5). The LSCM provided clear images of the HC and AC. Examples of uncolonised cells, HC and AC are shown in Figures 6.2 a, b & c respectively. The surface of the HC was smooth (Figure 6.2 b). The AC had looped hyphae similar to those of the HC, with small arbuscule-like protrusions on their surfaces (Figure 6.2 c). At no stage of the experiment were intercellular hyphae observed. The roots were 6.0 (±1.2), 13.6 (±3.0), 14.6 (±2.1), 30.7 (±2.7) and 45 (±5) % colonised at 4, 8, 12, 20 and 28 DAT respectively.

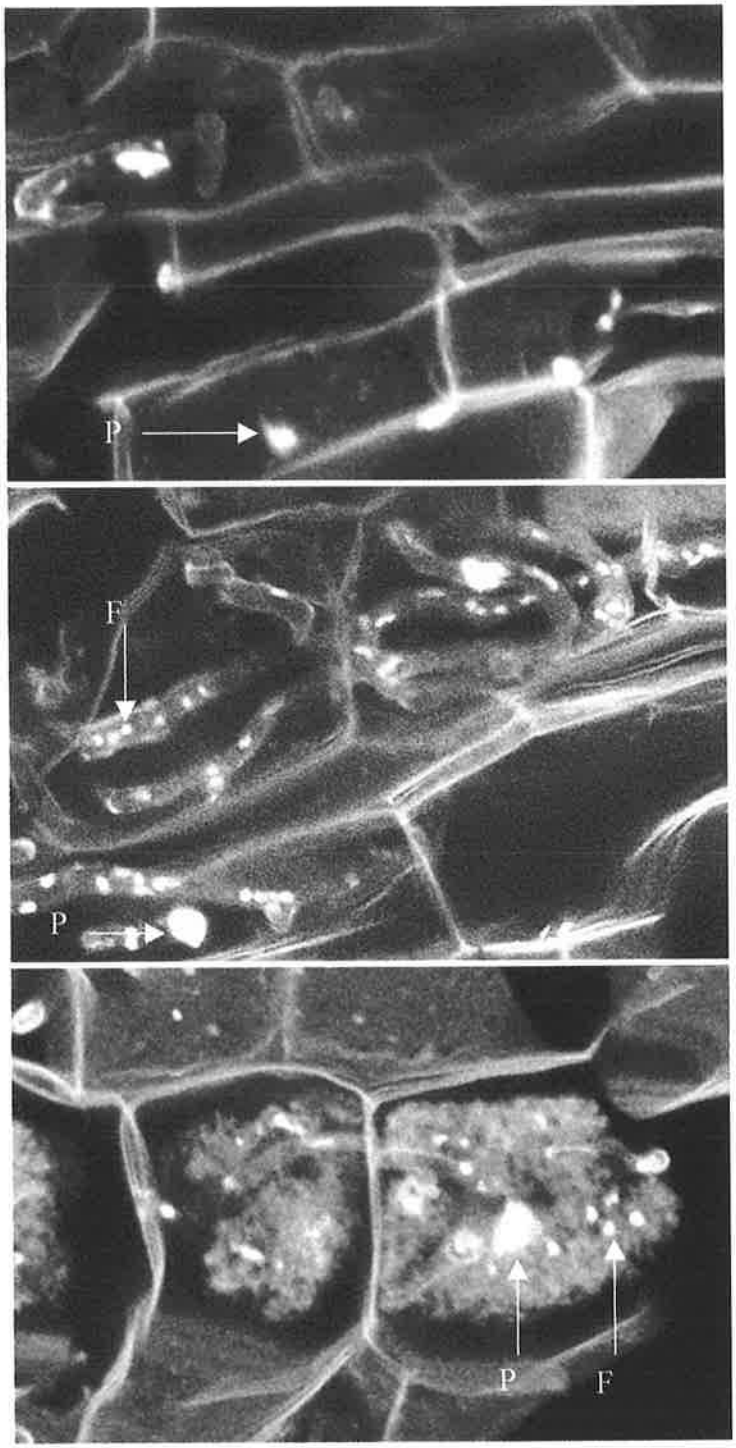


Figure 6.2 LSCM extended focus images of (a) uncolonised cells, (b) HC and (c) AC. Plant nuclei and fungal nuclei are labelled P and F respectively. Compare position of plant nuclei in (a), (b) and (c) relative to nearest cell wall. The horizontal dimension of the images is 144 μm.

6.3.2 Position and volume of the plant nuclei

The first AC and HC were observed 8 DAT, so measurements of nuclear size and position for 4 DAT were not made. The mean volume of the uncolonised and colonised cells of either inoculated or uninoculated plants at 28 DAT was 211048, 211773 and 216301 μm^3 respectively and was not significantly different ($p=0.95$). Thus, colonisation did not affect cell volume, which allowed direct comparison of nuclear position measurements. The distances from the centre of the plant nucleus to the nearest plant cell wall at 8, 12, 20 and 28 DAT in uncolonised cells of uninoculated plants and uncolonised cells and colonised cells of inoculated plants are given in Figure 6.3. The distance in uncolonised cells of both inoculated or uninoculated plants was considerably less that that in colonised cells. Furthermore, the distance in the cells, containing AC increased significantly between 8 and 12 DAT. This demonstrates that there is a major change in the position of the nucleus in cells colonised by AC. When comparing the distance from the centre of the plant nucleus to the closest plant cell wall in uncolonised cells of both inoculated and uninoculated plants there was no significant two-way interaction between inoculation treatment and harvest time. Therefore, only the main effects are presented (Table 6.1). The distance in uncolonised cells of both inoculated and uninoculated plants is given in Table 6.1. There was some variation in the distance with time, however there was not a consistent trend. Irrespective of harvest time, the distance from the centre of the plant nucleus in cells of uninoculated plants was slightly greater than in uncolonised cells of

inoculated plants, approximately 1.2 μm (Table 6.1). Conversely, the difference in the distance from the centre of the plant nucleus to the closest plant cell wall in colonised cells and uncolonised cells was in the range of approximately 2.5 to 10 μm (Figure 6.3). The small number of sections containing HC meant that it was not possible to measure changes in position with a sufficient sample size to allow for reliable statistical analysis of the data.

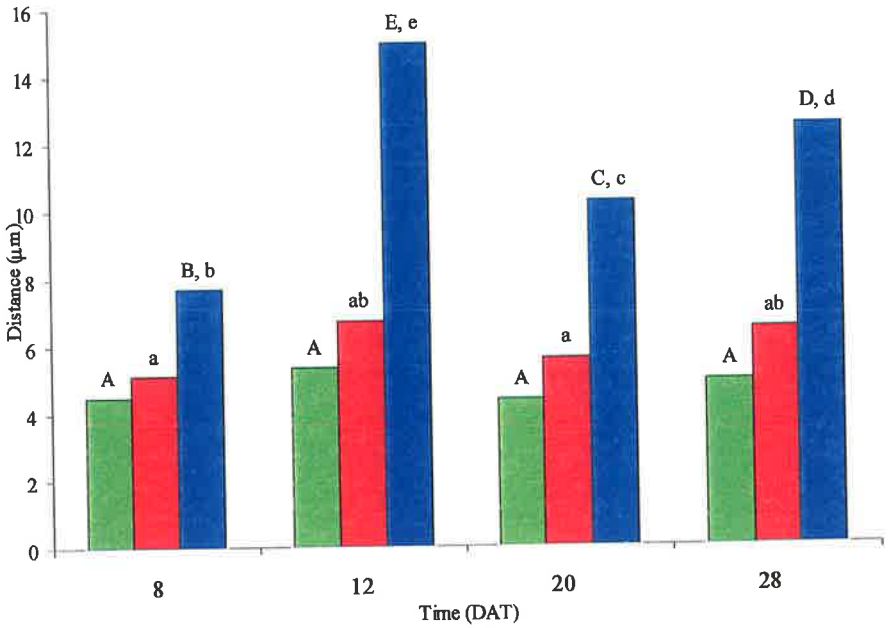


Figure 6.3 Mean distance from the centre of the plant nucleus to the nearest plant cell wall in uncolonised cells of uninoculated plants (■), uncolonised cells of inoculated plants (■) and colonised cells (AC) of inoculated plants (■). Because of the different levels of interaction (see Table 6.1) valid comparisons can only be made between means with uppercase and lowercase labels. Means with the same letter (uppercase or lowercase) are not significantly different when the least significant differences (LSD's) are compared ($P < 0.05$). ($n=10$).

Table 6.1 Mean distance from the centre of the plant nucleus to the nearest plant cell wall in uncolonised cells of inoculated and uninoculated plants and for each harvest time. Values followed by the same letter are not significantly different when the least significant differences (LSD's) are compared ($P < 0.05$). Comparisons between means can only be made for either inoculation treatment or harvest time. (n=10).

	Distance (μm)			
Treatment	**8 DAT**	**12 DAT**	**20 DAT**	**28 DAT**
Both inoculated and uninoculated plants	4.80^{a}	6.05^{c}	4.99^{ab}	5.69^{bc}

	Distance (μm)	
	Inoculated	**Uninoculated**
All harvest times	4.78^{a}	5.98^{b}

The volume of the plant nucleus calculated using different methods (see Figure 6.1) gave considerably different results (Table 6.2). The highest value was almost four times that of the lowest value. Given that the nuclei did not have a regular shape (see Figure 6.2), it was decided that stereological volume calculation would give the most reliable measurements.

Table 6.2 Volume of a nucleus from an uncolonised cell of an inoculated plant 28 DAT, calculated using a range of methods. Refer to Figure 6.1.

Method of volume calculation	**Volume μm^3**
Stereological	93.72
Sphere using (short axis rotation)	82.41
Sphere using (long axis rotation)	310.18
Prolate spheroid (short axis rotation)	128.19
Oblate spheroid (long axis rotation)	199.40

In colonised cells the plant nuclei were generally found to be lobed, although this was not the case for all nuclei. The volumes (calculated stereologicaly) of the plant nuclei at 28 DAT in the uncolonised cells of inoculated and uninoculated plants and colonised cells of inoculated plants are given in Figure 6.4. In cells colonised by AC the nuclei were larger (hypertrophied) compared to uncolonised cells of inoculated and uninoculated plants. This was also apparent in cells containing HC, but unfortunately the sample size was too small to obtain sufficient measurements for satisfactory statistical analysis.

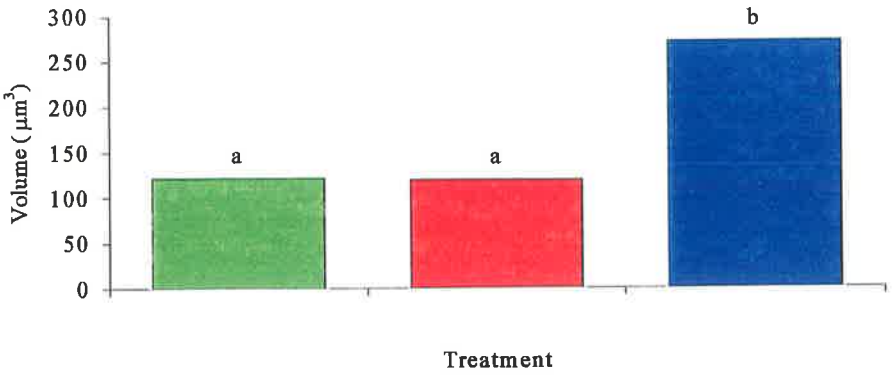


Figure 6.4 Volume of plant nuclei in uncolonised cells of uninoculated plants (■), uncolonised cells of inoculated plants (■) and colonised cells (AC) of inoculated plants (■). Means with the same letter are not significantly different when the least significant differences (LSD's) are compared ($P < 0.05$). (n=6).

6.3.3 Fungal nuclei

Spherical fungal nuclei were observed in the thick looped hyphae of the HC (Figure 6.2 b) and in the looped hyphae and thicker arbuscular branches of the AC (Figure 6.2 c). They were small; approximately 2 µm in diameter and the volume could not be accurately estimated from images collected using a z-step of 1.42 µm. There were up to six fungal nuclei per 30 µm of hyphae in the HC. The distribution of nuclei was irregular, although they were often paired.

6.4. Discussion

6.4.1 Development of the symbiosis

A. fistulosus colonised by *Glomus coronatum* formed a *Paris*-type AM with HC and AC as reported in Chapters 3 and 5. In *A. fistulosus* the smooth looped hyphae of the HC (Figure 6.2 b) were similar to those observed using LSCM in *Pa. quinquefolius* (Melville *et al.*, 1998) and *Lilium* sp. colonised by *Scutellospora calospora* (Dickson & Kolesik, 1999). The small arbuscule-like branches on the surface of the thick looped hyphae of the AC are morphologically similar to the smaller branches of arbuscules. The percent colonisation and rate of colonisation of the roots were similar to those reported in Chapter 5.

6.4.2 Position and volume of the plant nuclei

In cells containing AC, the position of the plant nuclei changed compared to uncolonised cells of both inoculated and uninoculated plants (Figure 6.3). At 8 DAT the distance from the centre of the plant nucleus to the closest cell wall was greater in colonised cells. The fact that there was no further increase in distance after 12 DAT in colonised cells suggests that the change in the nuclear position is a rapid process. Given the rate of morphological development of the *A. fistulosus*/*G. coronatum* symbiosis in nurse-pots (Chapter 5) this is not unexpected.

The data presented in Table 6.1 show that there was a marginal difference in the distance from the centre of the plant nucleus in uncolonised cells of inoculated and unincoulated plants (approxiamtely 1.2 μm). However, if we compare this difference in distance to that between colonised cells and uncolonised cells of either inoculated or uninoculated plants, the distance was approximately 2.5 to 10 μm (Figure 6.3). This implies that although there may be a small effect of colonisation of adjacent cells on the position of the nuclei, it is not nearly as marked as that seen in colonised cells.

Although it was not possible to make reliable measurements of the position of the plant nucleus in cells containing HC, some useful qualitative observations were made. It was apparent that nucleus moved to the centre of the plant cell (Figure 6.2 b) as with cells containing AC. Balestrini *et al.* (1992) observed no change in the position of the nuclei of *Al. porrum* in hypodermal cells containing entry coils. This difference between entry coils and HC with respect to the position of plant nuclei implies that there may be different interactions, for example, chemical signaling, between the fungi and the plant nucleus depending on the type of plant cell and coil.

The mechanism of the movement of the plant nuclei in cells colonised by arbuscules, AC and HC is not understood. Penetration of the cortical cell by the hyphae is a very invasive process. When a hypha colonising a cell invaginates the membrane of the cell, it may simply force the nucleus away from the periphery of

the cell. However, it is unlikely that the repositioning of the nucleus in colonised cells is due solely to mechanical movement of the nuclei by the fungi. For example, if a hypha were to enter the cell from a wall opposite the nucleus, it would be forced towards the opposite cell wall. But, given that the nucleus could be adjacent to any of the six walls, it is more likely that the hypha would enter the cell through a wall from which the nucleus was distant. Thus, movement of the nucleus due to mechanical movement by the hyphae is not likely to be the sole cause of nuclear shift.

The position of plant nuclei is closely linked to the spatial organisation of the plant cytoskeleton (Balestrini *et al.*, 1992). Bonfante & Perotto (1995) speculated that nuclear movement probably results from modifications in the organisation of the plant cytoskeleton as in pathogenic systems (see Koboyashi *et al.*, 1992). However, Genre & Bonfante (1998) found that the actin filaments reassemble in relation to the different spatial organisation of arbuscule-containing cells (*Arum*-type AM), particularly in relation to organelle movement, rather than nuclear movement as a result of modifications in the organisation of the plant cytoskeleton. This large rearrangement seen in the cytoskeleton of cells containing arbuscules is due to the invagination of the plant membrane caused by the development of intraradical fungal structures (Timonen *et al.*, 2001). This cytoskeletal rearrangement presumably occurs in cells containing AC or HC. However, this is yet to be determined.

The method of volume calculation of the plant nucleus has a large effect on the final volume calculated (Table 6.2). If it was assumed that the nucleus was spherical, the largest volume or the smallest, depending on whether Radius A or B was used in the calculations, was calculated. If it was assumed that the nucleus was a spheroid, then the choice of axis about which the spheroid is formed altered the volume calculated. Although stereology cannot provide an exact value for the volume of the nucleus, it does provide the most accurate value among the methods compared. This is because it does not assume that the nuclei are regular in shape nor that their surface is smooth (see Figures 6.2 a, b & c). The accuracy of stereological measurement can be improved by increasing the number of scans (in the z-axis) of the nucleus.

In cells containing AC the nuclei were hypertrophied (Figure 6.4), which is a common reaction of plants to microbial infection and has been related to unfolding of chromatin (Berta *et al.*, 1990). Similar hypertrophy was observed, but not quantified, in the *Paris*-type AM formed by *Li. tulipifera* colonised by *G. mosseae* (Kinden & Brown, 1975c). In AM systems there is no evidence that this hypertrophy is due to an increase in ploidy, but may be related to chromatin decondenstation (Berta *et al.*, 1990). Unfolding of the chromatin implies an increase in transcriptional activity within the cell.

6.4.3 Fungal nuclei

Bago *et al.* (1998b, 1999) studied nuclei in the external hyphae of AM fungi in great detail. However, the nuclei of AM fungi when forming HC and AC have not been studied. The nuclei in the thicker branches of the AC were distorted in a similar manner to those observed in arbuscules formed by *Gigaspora margarita* in *Al. porrum* (Bianciotto & Bonfante, 1992). From the observations described in this chapter, it is apparent that the nuclei in the HC are similar in shape, size and irregularity of position to those observed in external hyphae and intercellular hyphae of AM. This is not unexpected given the similar diameter of HC, external hyphae and intercellular hyphae.

6.5 Conclusions

LSCM has been used in the past to investigate the morphology, cytoskeleton and physiology of AM (for example Ayling *et al.*, 1997; Genre & Bonfante, 1998; Melville *et al.*, 1998 and Dickson & Kolesik, 1999). In this experiment LSCM allowed for more detailed and accurate measurements of positional and volume changes of plant nuclei than would have been possible using conventional light microscopy, or thin sectioning and observation using (two-dimensional) TEM. Although there is evidence to suggest that the plant cytoskeleton facilitates movement of the nucleus in colonised plant cells, at present, there is insufficient

evidence to determine what factors initiate or control nuclear shift in colonised cells.

The results presented in this chapter demonstrate that the arbusculate coils and probably cortical hyphal coils formed by *G. coronatum* have similar effects on their host plant (*A. fistulosus*) with respect to the position and size of the plant nuclei as cells of *Al. porrum* containing arbuscules. The increase in the volume of the nucleus in cells colonised by HC and AC is important because it implies that fungal/plant interactions similar (potentially decondensation of chromatin) to those observed in cells colonised by arbuscules are occurring. This may imply that there is signaling between the plant and the fungus.

CHAPTER 7 EFFECT OF SOIL P CONCENTRATION ON GROWTH AND PHYSIOLOGY OF *ASPHODELUS FISTULOSUS/GLOMUS CORONATUM* SYMBIOSIS

7.1 Introduction

Benefits to the plant as a result of the formation of AM are well described. The most widely studied benefit is that of improved acquisition of mineral nutrients, particularly phosphorus (P), and subsequent transfer to the plant. However, as emphasised in Chapter 1, there have been no detailed physiological studies of host responses to *Paris*-type colonisation, compared with the very large number done with *Arum*-type AM.

The addition of P to the soil can have a significant effect on the colonisation of roots by AMF. For example, small additions of P can result in a slight increase in colonisation (Bolan *et al.*, 1984) whereas with larger additions a reduction in colonisation is common (Smith & Read, 1997) and references therein). P addition can also influence AM at the structural level. For example, Bruce *et al.* (1994) reported a reduction in the abundance of arbuscules at higher soil P concentrations, but not in the number of entry points (see Chapters 1 and 4).

As demonstrated in Chapter 5, the time-course of development of the *A. fistulosus* and *G. coronatum* symbiosis is slower than that reported for *Arum*-type

AM. The slower rate of colonisation may result in a slower development of a growth response in *Paris*-type AM. However, this is yet to be determined.

Specifically, the aims of the work presented in this chapter were therefore:

I. To investigate the growth response of *A. fistulosus* colonised by *G. coronatum* at different soil P concentrations,

II. To investigate the effect of P addition to the soil on the development of colonisation and morphology of the *A. fistulosus/G. coronatum* symbiosis.

7.2 Materials and methods

7.2.1 Plant growth and phosphorus treatments

Pre-germinated *A. fistulosus* seeds (section 2.2.1) were planted into individual 70 mm diameter, plastic, non-draining pots (Polar Cup, Australia) containing 400 g of the 1:9 (w/w) mixture of the *G. coronatum* inoculum and soil/sand mix (section 2.3.1).

Five P treatments were included. Prior to planting the germinated *A. fistulosus* seeds, the P content of the inoculum and soil/sand mix was altered by mixing $NaH_2PO_4.H_2O$ solutions in the soil in each pot. The final soil P concentrations were 6.6 (no added P), 14.7, 30.7, 43.0 and 61.5 mg P kg^{-1} soil, referred to as P1, P2, P3, P4 and P5 respectively. The plants were grown in a glasshouse (section 2.2.3), watered three times a week and once a week starting two weeks after planting, the pots were fertilised with 5 ml of nutrient solution (section 2.2.2). For all treatments, non-mycorrhizal control pots were prepared in the same manner, the only difference being that the inoculum was replaced with soil and roots derived from non-mycorrhizal pot cultures.

Plants were harvested six and nine weeks after planting. Shoot and root dry weights were measured and shoot and root P contents were determined (section 2.4.3). A sub-sample of root material was cut into segments, cleared and

stained with trypan blue (section 2.4.4). The percentage of the root length colonised and the fractions of the colonised root length with external hyphae (EH), hyphal coils (HC) and arbusculate coils (AC) were determined using the magnified intersects technique (section 2.4.5.2).

7.2.2 Calculations and data analysis

Mycorrhizal growth responses (MGR) were calculated using the individual total plant dry weights (DW) of colonised (M) plants and mean weights of uncolonised (NM) plants: Equation 1.

$$\%\text{MGR} = \frac{\text{DW (M)} - \text{mean DW (NM)}}{\text{mean DW (NM)}} \times 100 \qquad \text{Equation 1.}$$

Mycorrhizal P responses (MPR) were calculated 1) using the individual P content of colonised plants and mean P content of uncolonised plants and 2) using equivalent shoot P contents: Equation 2. The rationale here was that as an unknown quantity of root P in the colonised plants is in the fungus and this may provide misleading results in terms of benefit to the plant. This complication is avoided if MPR is based on shoot P.

$$\%\text{MPR} = \frac{\text{P content (M)} - \text{mean P content (NM)}}{\text{mean P content (NM)}} \times 100 \qquad \text{Equation 2.}$$

Specific P uptake (SPU) between the six and nine week harvests was calculated using 1) the total plant P contents, and 2) shoot P contents, and individual and mean root dry weights: Equation 3. The rationale was again that root P would include P in intraradical AMF and inclusion might be misleading with respect to benefits to the plant.

$$\text{Specific P uptake rate} = \frac{\text{P content (9 weeks) - mean P content (6 weeks)}}{0.5(\text{RDW (9 weeks)} + \text{mean RDW (6 weeks)})} \quad \text{Equation 3.}$$

7.3 Results

7.3.1 Colonisation

The percentage of the *A. fistulosus* root length colonised decreased with increasing soil P concentration (Table 7.1). With increasing P addition to the soil, the percentage of the root length colonised decreased from 59.1% (P1) to 40.6% (P5) and from 56.5% (P1) to 36.7% (P5) at the six and nine week harvests respectively. Addition of P to the soil did not have a significant effect on the percent of the colonised root with EH, HC or AC at either harvest (Table 7.1). However, the difference between the percent of the colonised root containing HC relative to the percentage containing AC (HC-AC, the number of intersections containing AC subtracted from the number of intersections containing HC), increased significantly with increasing P (Table 7.1).

Table 7.1 Percent of root length colonised by *G. coronatum*, and percent of mycorrhizal root length containing EH, HC, AC and HC-AC of *A. fistulosus* at different soil P concentrations at the six and nine week harvests. Means followed by the same letter are not significantly different at the P<0.05 level. Valid statistical comparisons cannot be made between harvest times or between different colonisation features. (n=4).

	6 week colonisation					9 week colonisation				
	% Col.	EH	HC	AC	HC-AC	% Col.	EH	HC	AC	HC-AC
P1	59.1a	37.2	47.7	40.7	7.0ab	56.5a	39.7	42.0	37.0	5.0a
P2	55.0ab	50.0	56.0	51.0	5.0a	56.1a	52.0	41.5	41.7	9.8a
P3	47.2bc	49.2	54.5	47.5	7.0ab	41.4ab	54.5	52.2	36.0	16.3b
P4	44.2bc	50.0	54.7	42.2	12.5bc	33.2b	61.0	62.5	45.5	17.0b
P5	40.6c	52.2	58.2	44.2	14.0c	36.7b	50.0	46.7	31.0	15.8b
Sig.	<0.05	ns	ns	ns	<0.05	<0.05	ns	ns	ns	<0.05

7.3.2 Plant growth

The SDW and RDW for the six and nine week harvests are presented in Figures 7.1 a & b. At six weeks, the SDW of the colonised plants grown at P1 was significantly larger than that of the uncolonised plants. The same trend was apparent for RDW, but the difference was not significant. At P2 to P5 the SDW and RDW of the colonised plants were smaller than the uncolonised plants, but the difference in RDW at P5 was not significant. The maximum SDW and RDW

of the plants was achieved at P2. As the soil P was increased the difference between the SDW of the uncolonised and colonised plants decreased, as was also the case for the RDW.

Between the six and nine week harvests the SDW and RDW of the uncolonised plants grown in P1 did not change, but the colonised plants increased in weight dramatically (compare Figures 7.1 a & b). At nine weeks, both SDW and RDW of the colonised plants grown in P1 were much larger than the uncolonised plants. At P2 to P5, the SDW of colonised and uncolonised plants were not significantly different from each other or from the colonised plants at P1. The RDW of uncolonised plants grown in P2 were higher than those grown in P1. There was no increase with any further P addition. There was no consistent trend in the RDW of the colonised plants. As at the six week harvest, the increasing addition of P to the soil resulted in a decrease in the difference between the RDW of uncolonised and colonised plants at nine weeks. The data in Figure 7.1 were used to calculate the MGR, using Equation 1. At both the six and nine week harvests the MGR was positive at P1 and negative at P2 to P5 (Figure 7.2).

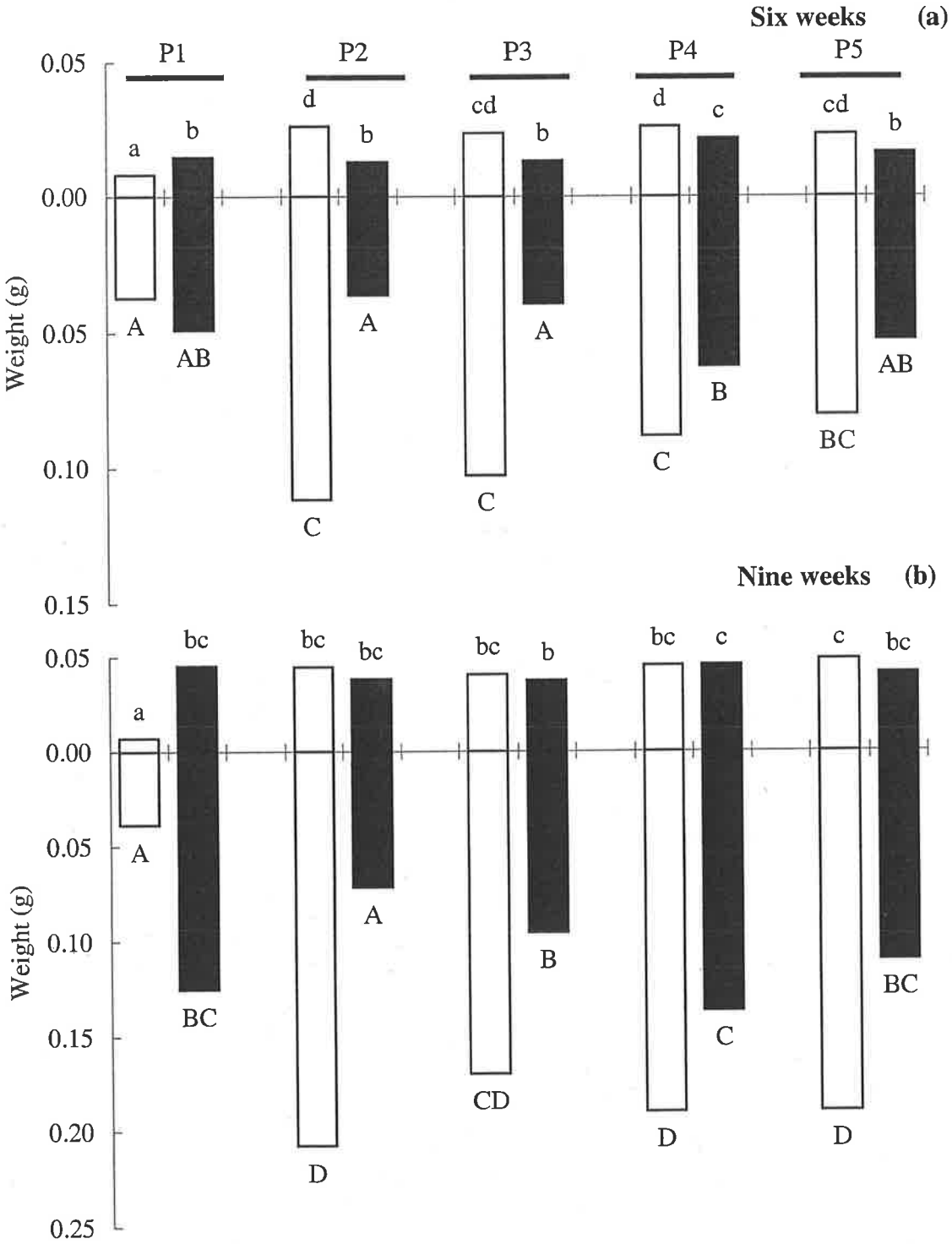


Figure 7.1 Shoot (above X axis) and root (below X axis) dry weights of uncolonised (□) and colonised (■) *A. fistulosus* at five soil P concentrations (a) at the six week harvest and (b) at the nine week harvest. Means followed by the same letter are not significantly different at the P<0.05 level. Valid statistical comparisons cannot be made between shoots and roots. (n=4).

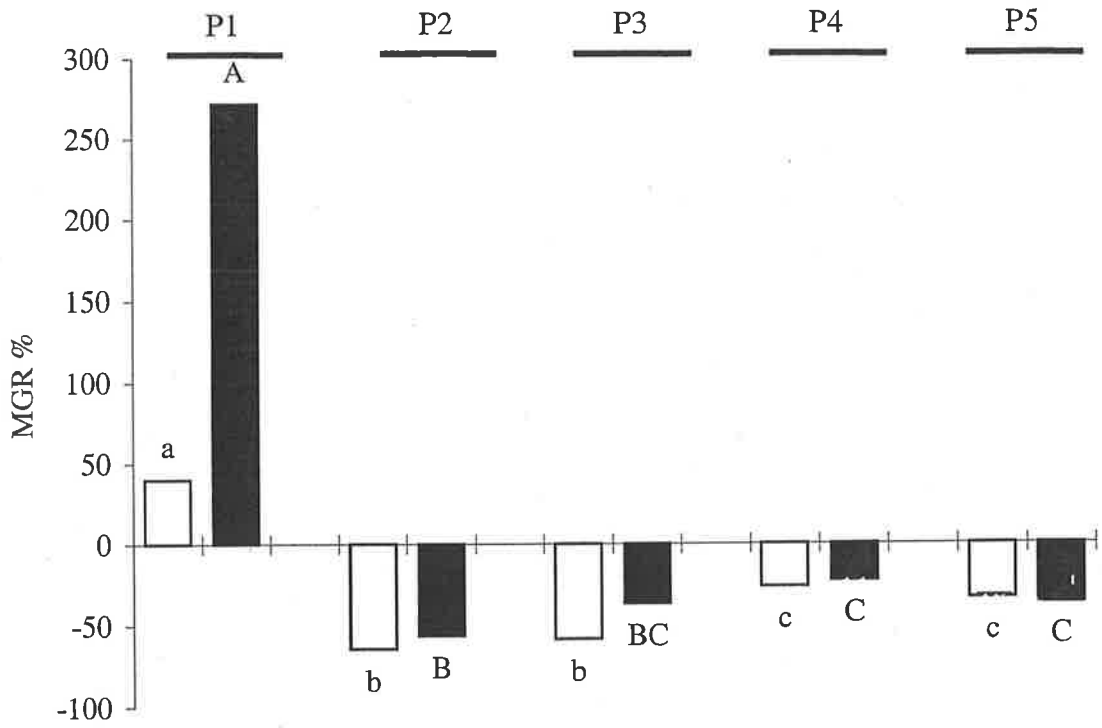


Figure 7.2 MGR (total dry weight) of *A. fistulosus* at the six (□) and nine (■) week harvests at five different soil P concentrations. Means followed by the same letter are not significantly different at the P<0.05 level. Valid statistical comparisons cannot be made between harvest times. (n=4).

7.3.3 Plant phosphorus

The shoot P concentration of both the colonised and uncolonised plants at six weeks tended to increase with increasing P addition to the soil (Figure 7.3 a). The only significant difference between colonised and uncolonised plants was observed at P5, with colonised plants having a greater P concentration than uncolonised plants. At P1 the root P concentration in uncolonised and colonised plants was the same. At P2 to P5 the colonised plants had significantly higher root P concentrations than the uncolonised plants. This difference decreased with increasing P addition.

At the nine week harvest, the P concentrations in the shoots of colonised plants in P1 were lower than those of uncolonised plants (Figure 7.3 b). At P2 and P3 the shoot P concentrations in the colonised plants were higher than in the uncolonised plants, but with further addition of P to the soil there were no significant differences. The root P data at this harvest were very similar to those observed at six weeks in both concentrations and trends.

P contents of the tissues (ie the products of dry weights and P concentrations) were used to calculate the MPR for total plant P content (Figure 7.4 a) and shoot P content (Figure 7.4 b), using Equation 2. Both formulations showed significantly higher mycorrhizal P responses at lower soil P concentrations compared to the higher soil P concentrations. The MPR was

greatest at the nine week harvest at P1 and in most cases was higher at the nine week harvest compared with the six week harvest.

The SPU of the uncolonised and colonised plants between the six and nine week harvest were calculated, again using the total plant P content (Figure 7.5 a) and the shoot P content (Figure 7.5 b), using Equation 3. Using both methods of calculation, SPU of uncolonised and colonised plants at P1 and P2 were not significantly different, although there was a clear trend towards greater SPU in colonised plants. At P3 to P5 the SPU of the colonised plants was significantly greater than that of the uncolonised plants and then became higher with increasing soil P when total plant P was used but not when shoot P only was used.

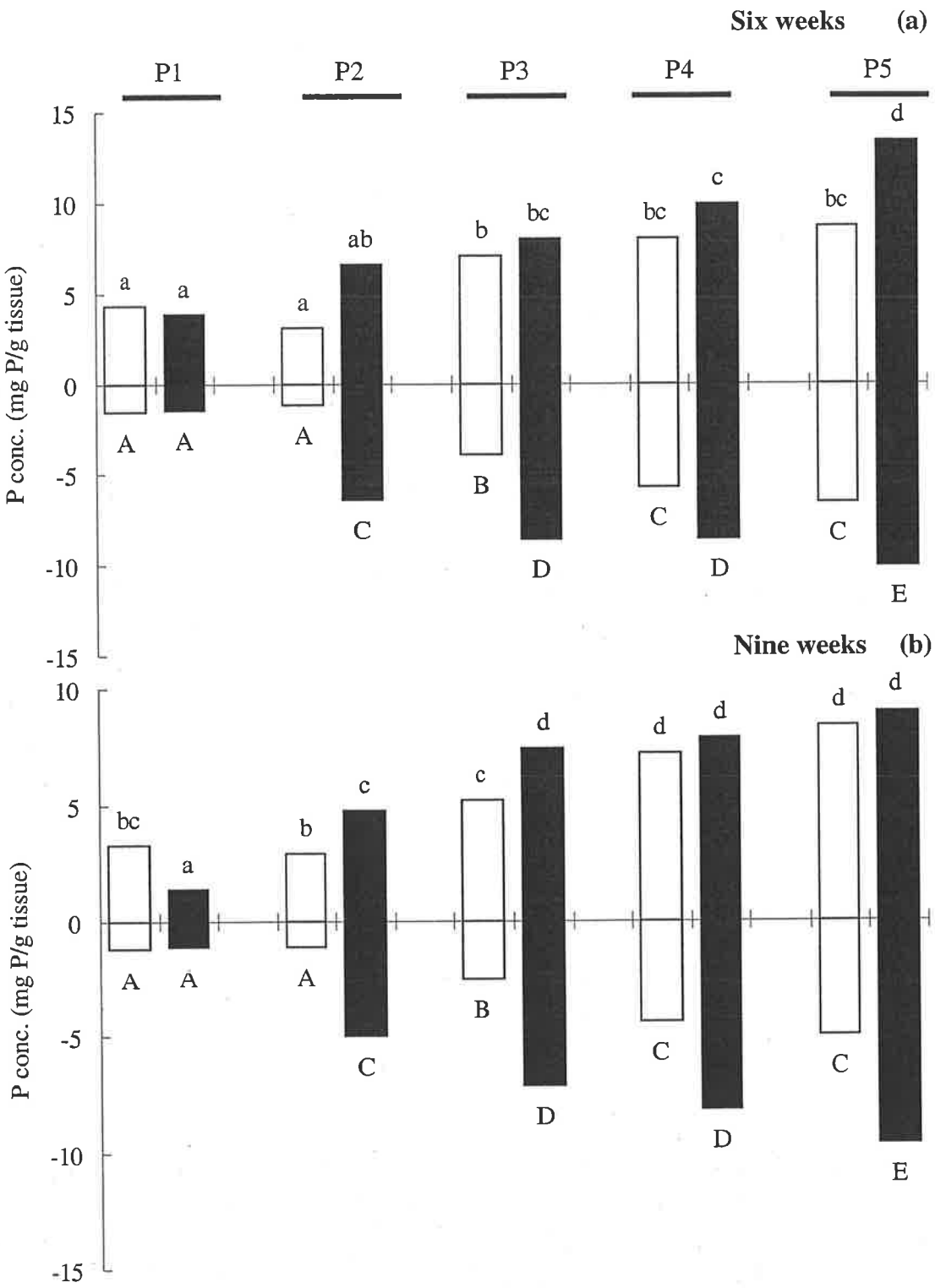


Figure 7.3 Shoot (above X axis) and root (below X axis) P concentration in uncolonised (□) and colonised (■) *A. fistulosus* at five soil P concentrations (a) at the six week harvest and (b) at the nine week harvest. Means followed by the same letter are not significantly different at the $P<0.05$ level. Valid statistical comparisons cannot be made between shoots and roots. (n=4).

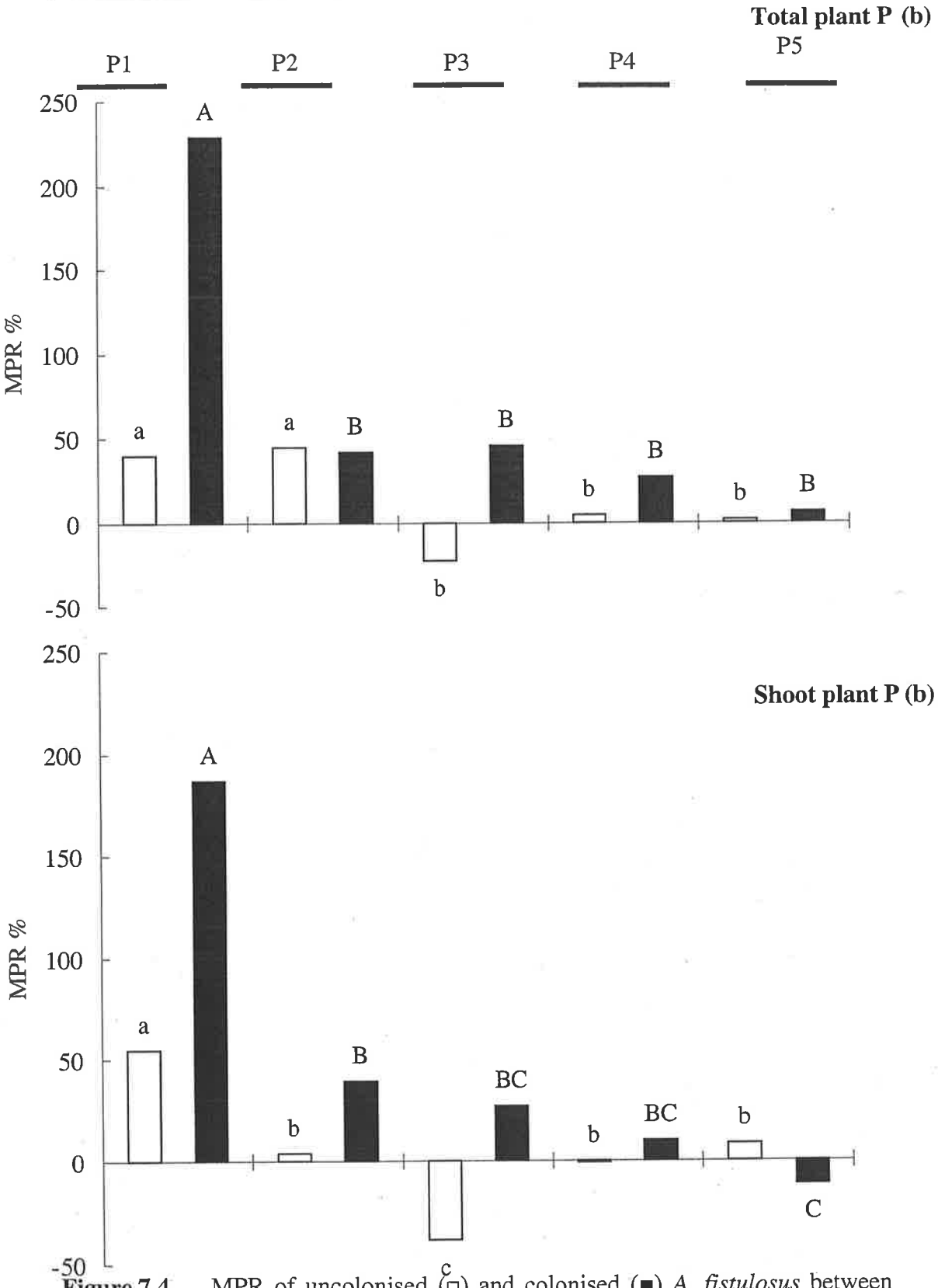


Figure 7.4 MPR of uncolonised (□) and colonised (■) *A. fistulosus* between the six and nine week harvests calculated using **(a)** total plant P content and **(b)** shoot P content. Means followed by the same letter are not significantly different at the $P<0.05$ level. (n=4).

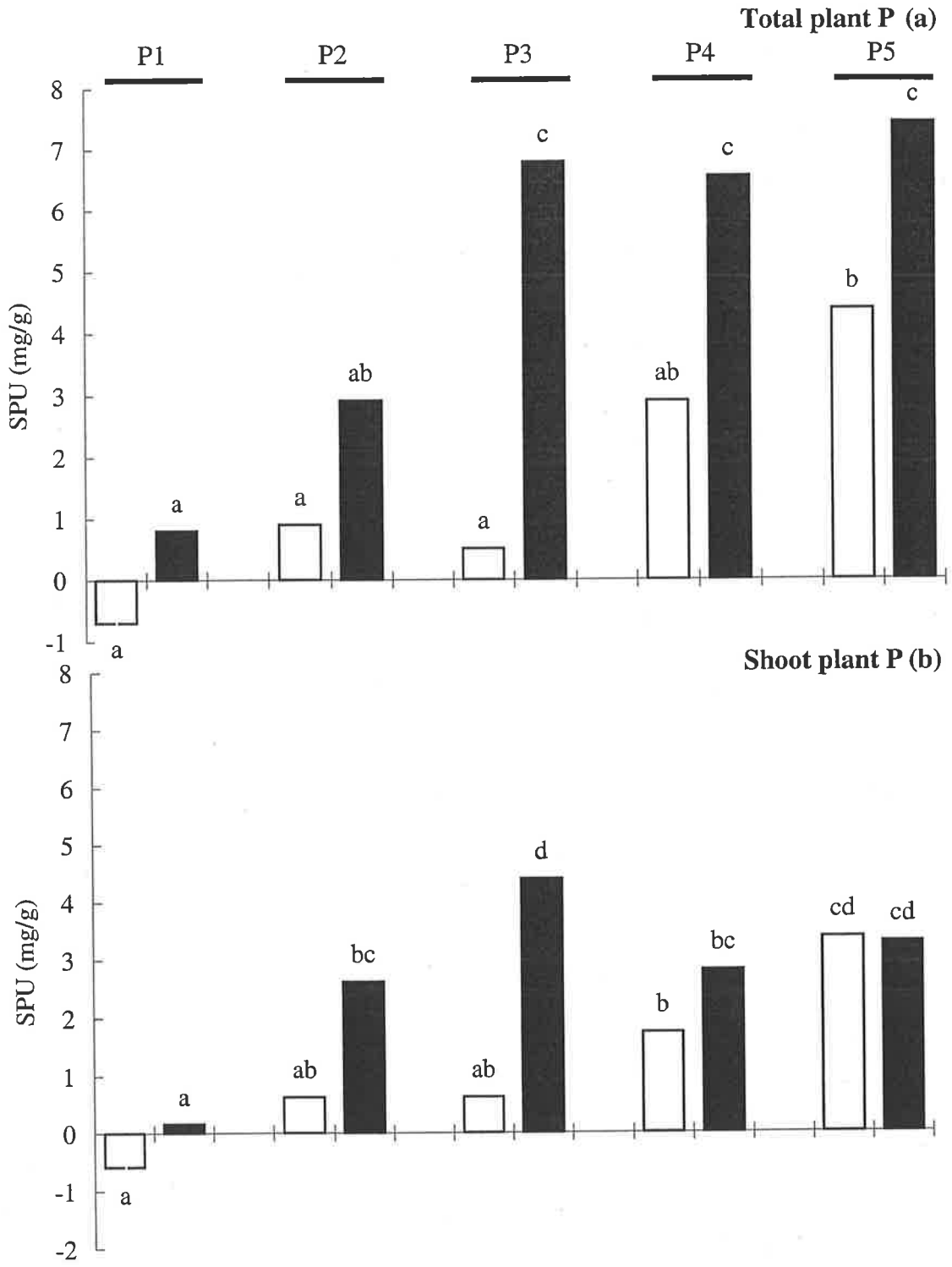


Figure 7.5 SPU of uncolonised (□) and colonised (■) *A. fistulosus* between the six and nine week harvests calculated using (a) total plant P content and (b) shoot P content. Means followed by the same letter are not significantly different at the $P<0.05$ level. (n=4).

7.4 Discussion

7.4.1 Colonisation

A. fistulosus and *G. coronatum* formed a *Paris*-type AM, as reported previously (See Chapters 3, 5 and 6). The addition of P to the soil resulted in a significant reduction in the percentage of the root length colonised. It is difficult to make comparisons to effects of soil P concentration of percent colonisation of *Arum*-type AM, as a wide range of effects have been reported. For example, Baon *et al.* (1992) reported a large reduction in the length of *Triticum aestivum*, *Hordeum vulgare* and *Secal cereale* roots colonised by indigenous soil AMF with increased soil P concentration. Dickson *et al.* (1999a) found that there was a slight reduction in the percentage of *Al. porrum* roots colonised by *G. coronatum*, the percent colonisation was generally lower in soils with a higher P concentration except at the first harvest where it was reversed. Thomson *et al.* (1986) found that the percentage of *T. subterraneum* root length colonised decreased with increased soil P concentration, however, at a greater rate with *Scutellospora calospora* compared to *G. fasiculatum*, highlighting the importance in fungal differences.

The addition of P to the soil did not significantly influence the proportion of the colonised root length containing EH, HC and AC. As discussed in Chapters 1 and 4, for *Arum*-type AM there have been differing reports of the influences of P addition on morphology (Amijee *et al.*, 1989; Smith & Gianinazzi-Pearson,

1990; Bruce *et al.*, 1994). Bruce *et al.* (1994) observed a reduction in the number of arbuscules but not entry points in *C. sativus* colonised by *G. intraradices*. Conversely, Amijee *et al.* (1989) reported a decrease in the number of entry points with increased P addition in *A. porrum* colonised by *G. mosseae*. These differences may be a reflection of the plant species, fungal species or experimental conditions used. In the present experiment there were no apparent effects on morphology when each structure is considered separately. However, at both the six and nine week harvests, as the soil P was increased, the number of intersections containing AC decreased relative to HC (ie, HC-AC increased, Table 7.1). This suggests a possible functional difference between the structures. However, at this stage there is insufficient evidence to make firm conclusions on the significance, functional or otherwise, of these small changes. Furthermore, it is not sensible to make generalisations about the *Paris*-type based on one experiment with one plant and fungus combination, hence, there is a need for further research before more firm conclusions can be made.

7.4.2 Plant growth

The majority of P response experiments conducted to date have used crop plants, which mostly form *Arum*-type AM (Smith & Smith, 1996). To my knowledge this is the first detailed P response experiment conducted using a *Paris*-type AM and it provides insight into the functioning of what is apparently the most common morphological type of AM.

In this experiment, at six weeks and P1, there was a large positive benefit due to colonisation, similar to many *Arum*-type AM responses in that the colonised plants had a significantly larger SDW than the uncolonised plants. However, there was no significant difference in the RDW between colonised and uncolonised plants. Comparing the SDW and RDW of the uncolonised plants in P1 at both the six and nine week harvests shows that there was very little change between the two harvest times, whereas the colonised plants grew considerably between the two harvests. This is reflected in the MGR at P1, which was positive at the six week harvest, but much lower than at the nine week harvest. The MGR at P1 and six weeks was also less than that reported for *Al. porrum* colonised by the same fungus (MGR=112%) (Dickson *et al.*, 1999a). This difference in MGR may be a due to the slower growth of *A. fistulosus* compared to *Al. porrum* or to the slower rate of colonisation of *A. fistulosus*. These results highlight the importance of AMF for *A. fistulosus* in low P soils for continued growth over the time period assessed. Given the poor growth of uncolonised *A. fistulosus* at P1, it would be interesting to determine if it is obligately mycorrhizal under low P conditions, and the effects of colonisation on fecundity and competition.

At six weeks, P2 to P5, the SDW and RDW of the colonised plants were significantly lower than those of the uncolonised plants (with the exception of RDW at P5). At P2 to P5 concentrations, the root P concentrations of colonised plants were considerably higher than in the uncolonised plants. The shoot P concentrations of the colonised plants were either equal to or greater than those of

the uncolonised plants; however, the SDW of the colonised plants were significantly less than that of the uncolonised plants. At these higher soil P concentrations, *A. fistulosus* may be able to access enough P to satisfy its nutritional requirements without the contribution of the fungus. Thus, the fungus may have become superfluous, and potentially a cost to the plant. This is consistent with the suggestion of Johnson *et al.* (1997), that increasing availability of a limiting soil resource can convert balanced mutualistic relationships into less balanced ones, of which some can be parasitic.

MPR and SPU were calculated using both the total plant P content and the shoot P content. This is important as calculations based on total "plant P content" may be misleading if some of the total plant P is sequestered within the fungus. Despite the unknown amount of sequestered P, similar trends were observed when using both methods of calculation. At low soil P (P1), at both harvest times, the MPR was greater than at higher soil P concentrations except at P2, 6 weeks calculated on a total plant P basis. Of particular interest is the high MPR at P1 at the nine week harvest. This again emphasises the importance with respect to P acquisition for *A. fistulosus* of forming AM in low soil P conditions. The SPU (based on total plant P) of colonised plants was greater than that of the uncolonised plants in all P treatments, although differences were only significant at P3, P4 and P5. As soil P concentration increased a plateau in the SPU was reached, at lower P in the colonised plants (P3) than in the uncolonised plants (P4). The higher SPU in the mycorrhizal plants, given lower RDW than

uncolonised plants, is further evidence that much of the P is taken up and transferred via the AMF, that is, a "hidden addition" to the below-ground biomass of the AM plants. In other words, part of the higher SPU in the colonised plants is in fact due to P uptake via the fungal hyphae. Although the concentration within the mycorrhizal roots is higher than the non-mycorrhizal roots, there is not evidence to show how much of the P is sequestered within the fungus. The issue of sequestration of P within the fungal biomass within the roots is complicated. For example, the SPU at P4 and P5 was higher in the mycorrhizal plants compared to the non-mycorrhizal plants when calculated on a total plant P content (Figure 7.5 a) whereas, the mycorrhizal and non-mycorrhizal plants have equivalent SPU when calculated on the basis on shoot P content (Figure 7.5 b). This may reflect a sequestration of P within the roots or simply that the mycorrhizal roots had a higher P concentration than the non-mycorrhizal roots. Smith, Jakobsen & Smith (2000) discussed this issue and concluded that it is important that retention of P within AMF within and on the surface of roots should not be ignored.

In studies of *Arum*-type AM, between 4 and 20% of net photosynthate is transferred to the AMF and used in production of vegetative and reproductive structures, and in respiration to support growth and maintenance, including nutrient uptake (Smith & Read, 1997). This supply of photosynthates to the fungus is often compensated for by reduced root production and sometimes alterations in root shoot ratios. Similar compensating processes were apparently

operating in the *Paris*-type symbiosis between *A. fistulosus* and *G. coronatum*, as seen in the reduced RDW at the nine week harvest, P2 to P5. Given that the plants had similar SDW irrespective of colonisation, it can be concluded that in higher P soils there was no apparent net benefit in terms of plant growth in forming AM under these conditions. However, if another pressure/limitation is imposed upon the plant, such as a pathogen or drought, the fungus may be able to confer a further benefit to the plant (see West, Fitter & Watkinson, 1993a, b and Marler, Zabinski & Callaway, 1999). Given that *A. fistulosus* is a perennial herb, this is of particular importance. Conversely, the reduction in the RDW may limit the acquisition (by the roots) of nutrients other than P, that the hyphae of AMF do not readily take up and transfer to the plant. This is a case where colonisation may result in either long-term gains or losses. The balance between costs and benefits, which will depend on the environmental characteristics of each situation and may change with time.

The negative MGR (at P2 to P5) may in part be due to a C drain and can be considered a "cost of establishment" of the symbiosis. The percentage of the root length colonised was relatively low compared to other studies where C drains have been observed (see Graham & Eissenstat, 1998). However, in *Paris*-type AM the fungal biomass per unit root length of colonised root is likely to be higher than in *Arum*-types. In *Arum*-type AM arbuscules are not often formed in each consecutive cell within the cortex, but rather in every second or third cell, whereas in *Paris*-types, extensive coils are formed in consecutive cells. According to the

calculations of Dickson & Kolesik (1999), representative arbuscules and hyphal coils have similar volumes and hence biomass. Thus, in the *Paris*-type the length of the root colonised might contain the same fungal biomass and exert the same C drain as an *Arum*-type AM with a larger length of the root colonised. This does not take into account the density of colonisation, the surface area of arbuscules versus hyphal coils, or the site of carbon transfer and these factors need to be investigated further.

7.5 Conclusions

A. fistulosus is of Mediterranean origin and is a common weed in southern Australia and elsewhere in the world. In southern Australia at least, sites invaded by *A. fistulosus* are often unfavorable in terms of plant growth, thus successful formation of symbiotic AM may provide a competitive advantage over invasive non-mycorrhizal species colonising these sites (O' Connor, 2001). The results presented here suggest that in soils with low P, *A. fistulosus* will benefit from forming AM both in early and late stages of growth, and colonisation may in fact provide an essential advantage in terms of competition and success. Conversely, in higher P soils, *A. fistulosus* may benefit only in later stages of growth and possibly only if a further stress is imposed upon the plant. The delay in apparent benefit may be of particular importance given that *A. fistulosus* is a perennial herb. Thus the longer term effects of colonisation need to be considered. The results here support the observation that the *Paris*-type tends to be found in

longer-lived roots than the *Arum*-type (Brundrett & Kendrick, 1990a, b). Furthermore, given the large volume of hyphal coils (Dickson & Kolesik, 1999) and their long life-span, they may provide an important site for storage of P within roots, especially perennial plants. This would certainly provide a competitive advantage in some situations. The importance (in terms of competitiveness) of this apparent delay in potential benefit at higher soil P will depend on the growth and competitiveness of other plants in field communities. *A. fistulosus* is a useful plant to study the physiology and ecology of AM (particularly *Paris*-type AM) given its large change in growth with and without colonisation by AMF over a narrow range of soil P concentrations (P1 and P2).

CHAPTER 8 THE INFLUENCE OF FUNGAL IDENTITY ON ARBUSCULAR MYCORRHIZAS FORMED BY *LYCOPERSICON ESCULENTUM*

8.1 Introduction

The work presented in this chapter was done in conjunction with another PhD student in the Department of Soil and Water, Adelaide University, Ms L-L Gao, part of which has been published in the *New Phytologist*:

Cavagnaro TR, Gao L-L, Smith FA, Smith SE. 2001. Morphology of arbuscular mycorrhizas is influenced by fungal identity. *New Phytologist* **151:** 469-476. (Bound at rear of thesis).

As discussed in Chapter 1, studies of different plant and fungal combinations have suggested that AM morphological type is dependent on the plant species (Smith & Smith, 1997). Unexpectedly, when investigating the interactions between AMF species and a mycorrhiza-defective mutant of *Ly. esculentum* Mill. (Gao, Delp & Smith, 2001), Ms Gao observed in a preliminary experiment, considerable variation in the extent of colonisation and internal morphology of the wild-type controls. Investigations of details of the internal morphology and effects of fungal identity on the formation of different morphological types in the wild-type host were not part of the original scope of Ms Gao's project and are of particular

relevance to this thesis. Ms Gao grew the plants as part of a major experiment and I assisted with harvesting and sample preparation. I then analysed the samples in detail to assess the contribution of fungal identity on AM morphology. We are both authors of the resulting paper.

Specifically, the aims of the work presented in this chapter were therefore:

I. To determine whether there are any differences in the internal morphology of AM formed by *Ly. esculentum* when colonised by six species of AMF,

II. To collect quantitative data and qualitative observations on any morphological differences observed.

8.2 Materials and methods

8.2.1 Plant growth, harvesting and colonisation

Al. porrum nurse-pots were established (note: different methods from Chapters 5 and 6) by growing seedlings with inoculum of six different AM fungal species (*Gigaspora margarita*, *Glomus coronatum*, *G. intraradices*, *G. mosseae*, *G. versiforme*, *Scutellospora calospora*). Briefly: pre-germinated *Al. porrum* seeds were planted in plastic, free draining pots containing 700 g of a 1:9 (w/w) inoculum/soil mixture. The inoculum was derived from pot cultures of each of the fungal species and the soil was a 1:9 (w/w) mixture of soil/washed sand. The sand was a 1:3 (w/w) fine/coarse sand mixture. The soil used depended on the

individual fungal species (see section 2.3.1). Eight weeks after planting of the nurse-pots, *Ly. esculentum* (Mill.) cv 76R seedlings (pre-germinated and grown in seedling trays for 16 days in sterile sand), were transplanted into the nurse-pots. Four *Ly. esculentum* plants were transplanted into each of three replicate nurse-pots.

The plants were grown in a growth chamber (14 hr photoperiod; 500 μmol m^{-2} s^{-1} proton flux density and temperatures of 18 and 25° C dark/light phases respectively) and watered with half-strength modified Long Ashton solution minus P (section 2.2.2), every day for the seedling trays, and three times per week for the nurse-pots.

Ly. esculentum plants were harvested 42 days after planting (section 2.4.1). Fractional colonisation of the roots was determined using the magnified intersects technique (section 2.4.5.2). Because of the importance of clearly distinguishing the different features, a conservative approach to scoring was adopted, whereby only unequivocally recognisable structures were recorded. Structures scored were, external hyphae (EH), hyphal coils (HC), arbusculate coils (AC), intercellular hyphae (IH), arbuscules (A) and vesicles (V).

8.3 Results

8.3.1 Total colonisation

The percent of the total root length colonised in *Ly. esculentum* varied greatly with species of AM fungus (Figure 8.1 a). Colonisation ranged from 40 to 85%, with the exception of *G. coronatum* (13.7%), which was considerably lower than that observed in the preliminary experiment (Gao, personal communication). Eight weeks after planting, the mean percentage of the root length of *Al. porrum* (nurse-plants) colonised by *G. intraradices*, *G. mosseae*, *G. coronatum*, *Gi. margarita*, *S. calospora* and *G. versiforme* was 83.8^{b}, 85.2^{b}, 41.1^{a}, 77.1^{b}, 88.6^{b} and 82.1^{b} respectively. Means followed by the same letter are not significantly different at the $P<0.05$ level.

8.3.2 Colonisation morphology

There were very large differences between the patterns of colonisation by different fungi in *Ly. esculentum*. The fractions of the colonised length occupied by EH, HC, AC, IH and A are presented in Figure 8.1 b to f. Vesicles were only observed in *Ly. esculentum* plants colonised by *G. intraradices*, *G. mosseae* and *G. versiforme*; the mean fraction of the colonised root length containing vesicles was 12^{b}, 21.7^{c} and 2.3^{a} for the three fungi respectively. Typical infection units

formed by the six different fungal species with *Ly. esculentum* are presented in Figure 8.2 a to f.

When colonising the roots of *Ly. esculentum*, *G. intraradices*, *G. mosseae* and *G. versiforme* all formed *Arum*-type mycorrhizas, with characteristic IH and A. *S. calospora*, *G. coronatum* and *Gi. margarita* all formed the *Paris*-type, with intracellular HC and AC. *G. coronatum* and *Gi. margarita* both formed some IH, but at very low frequencies. *G. mosseae* formed some HC, but these were entry-type coils in the outermost cell layers. The results obtained confirm the observations made in the preliminary experiment which also included *G. fasciculatum* and *G. etunicatum*. These two fungi both formed *Arum*-type AM. *Al. porrum* nurse-plants colonised by the different fungal species all formed the *Arum*-type. A typical arbuscule formed by *G. intraradices* when colonising *Al. porrum* is presented in Figure 8.3.

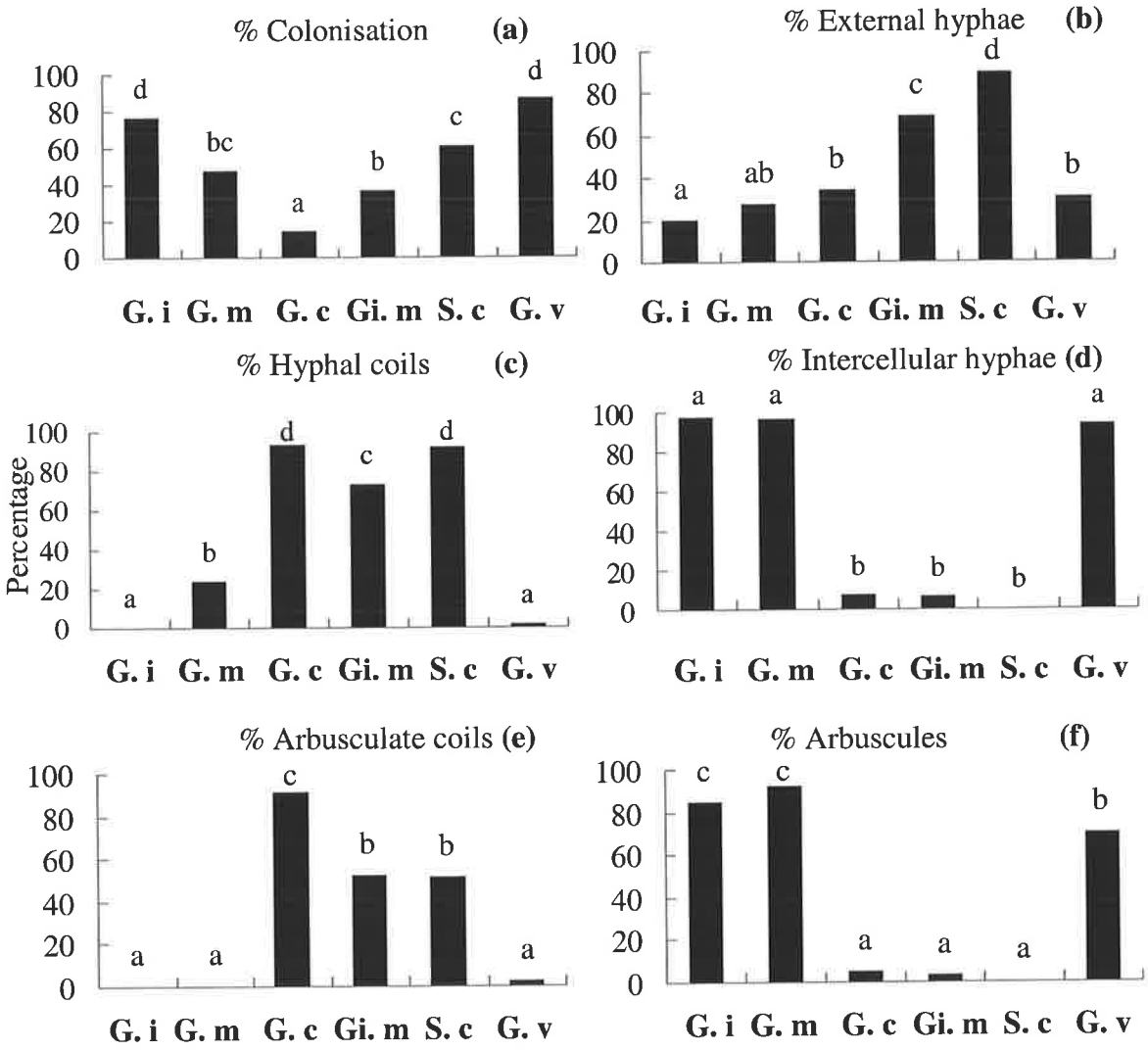
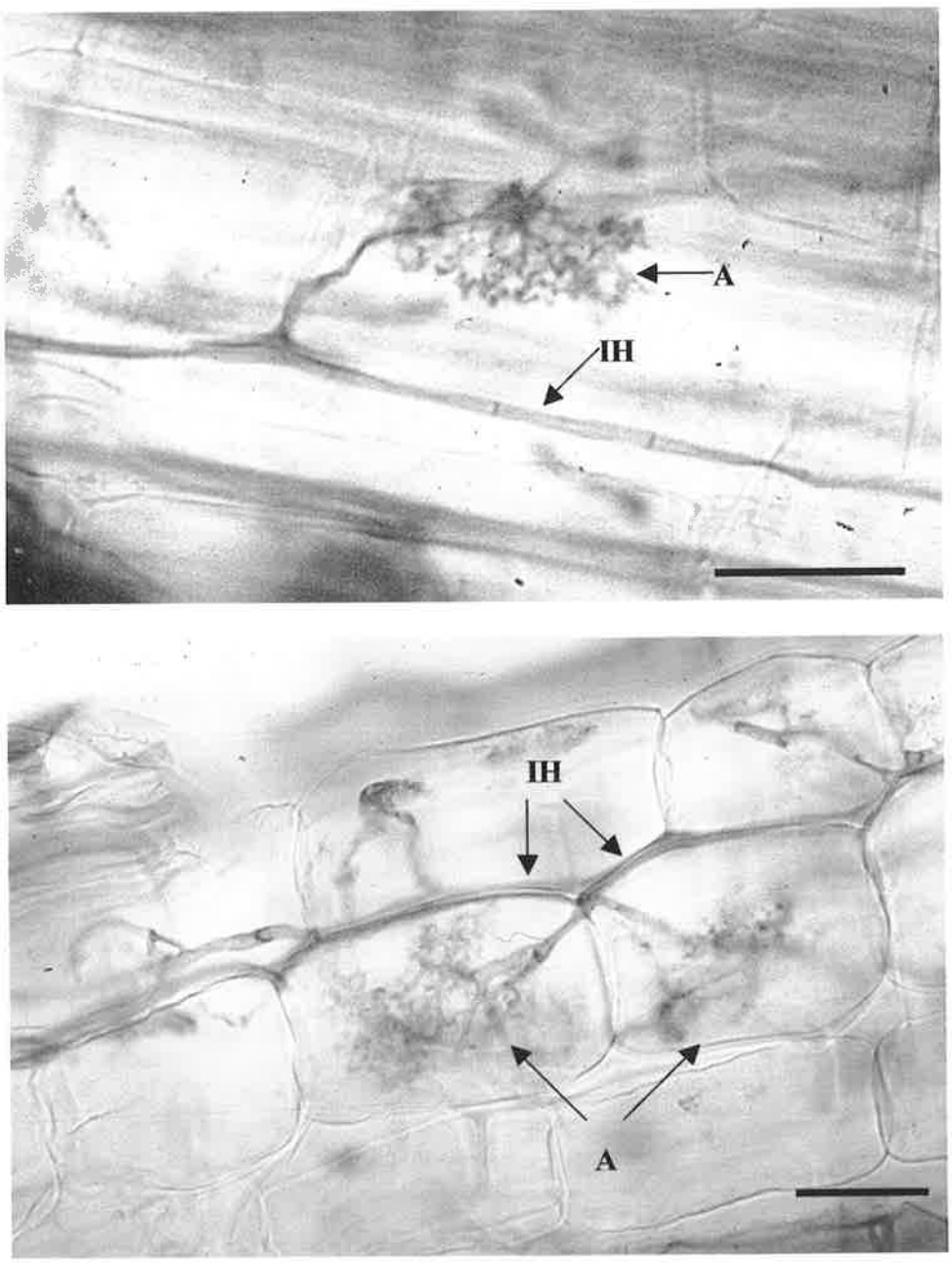
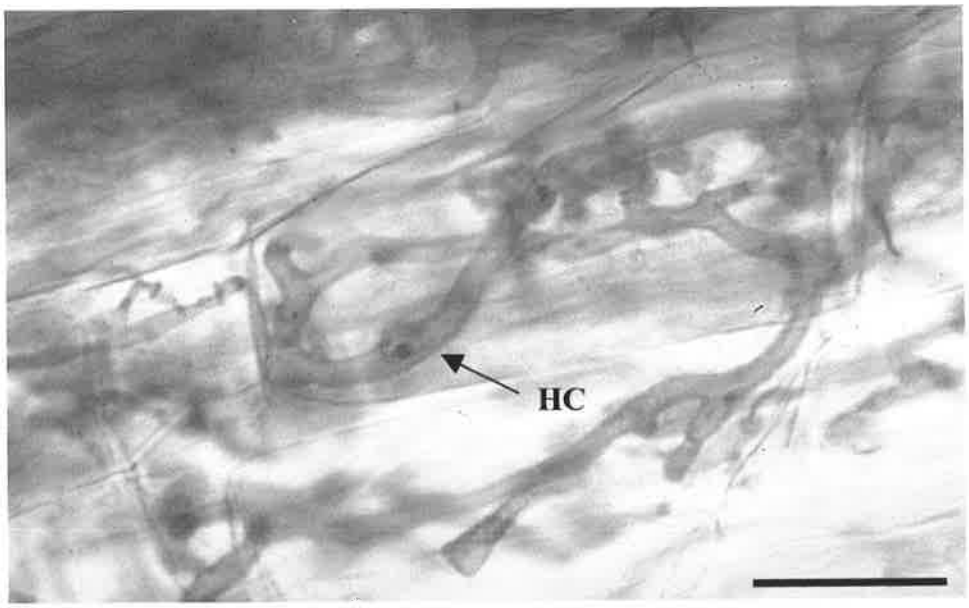


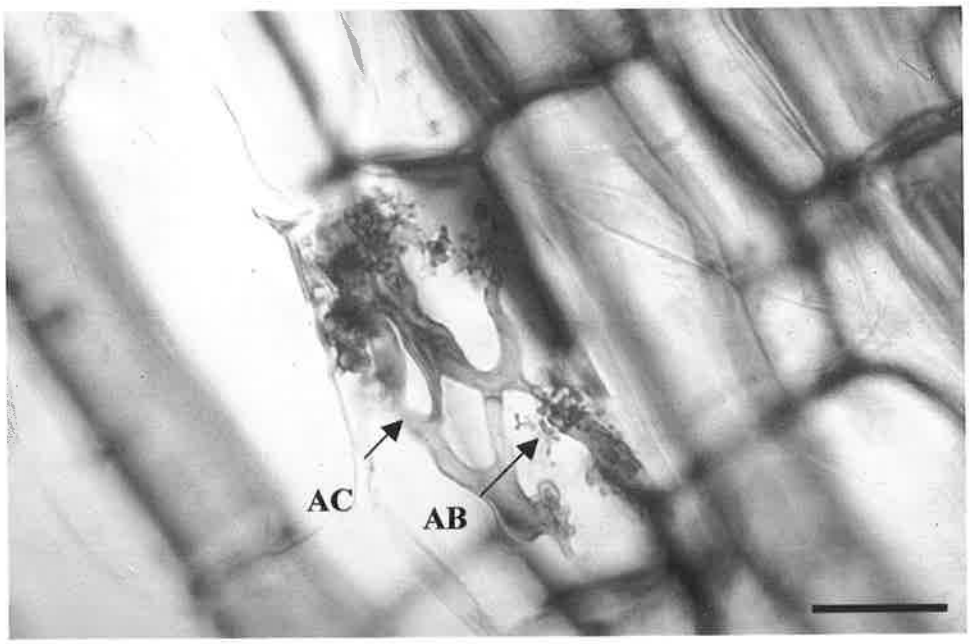
Figure 8.1 (a) Total percentage of the root length colonised and the percentage of the colonised root length containing (b) external hyphae, (c) hyphal coils, (d) intercellular hyphae, (e) arbusculate coils and (f) arbuscules. G.i = *G. intraradices*, G.m = *G. mosseae*, G.c = *G. coronatum*, Gi. m = *Gi. margarita*, S.c = *S. calospora*, and G.v = *G. versiforme* . Means with the same letter are not significantly different at the 5% level. (n=3).



Caption on Page 163



(c)



(d)

Caption on Page 163

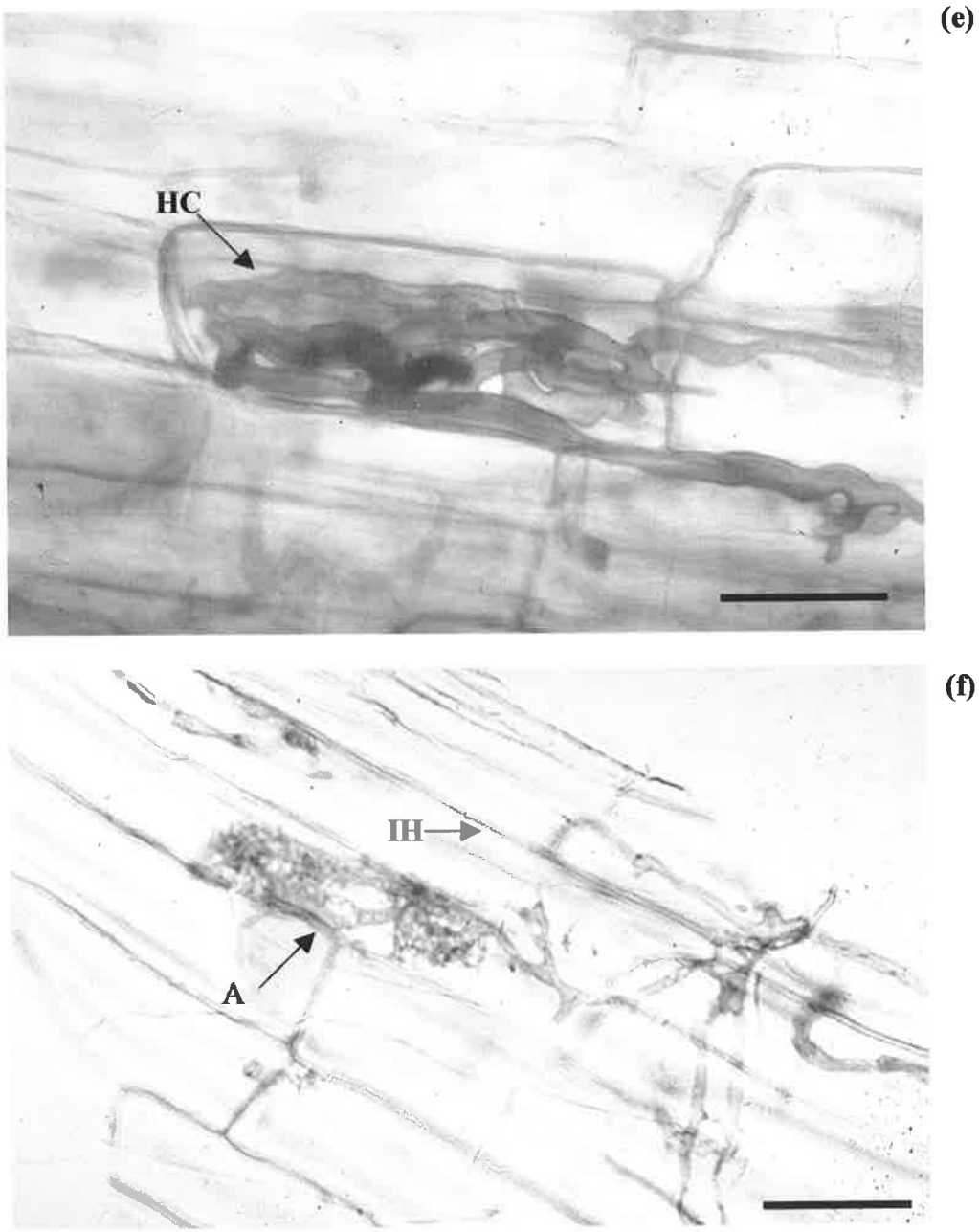


Figure 8.2 Typical infection units formed by (a) *G. intraradices*, (b) *G. mosseae*, (c) *G. coronatum*, (d) *Gi. margarita*, (e) *S. calospora* and (f) *G. versiforme* in association in *Ly. esculentum*. Note: A = arbuscule, IH = intercellular hyphae, HC = hyphal coil, AC = arbusculate coil and AB = arbuscule-like branches. Bars = 160 μm.

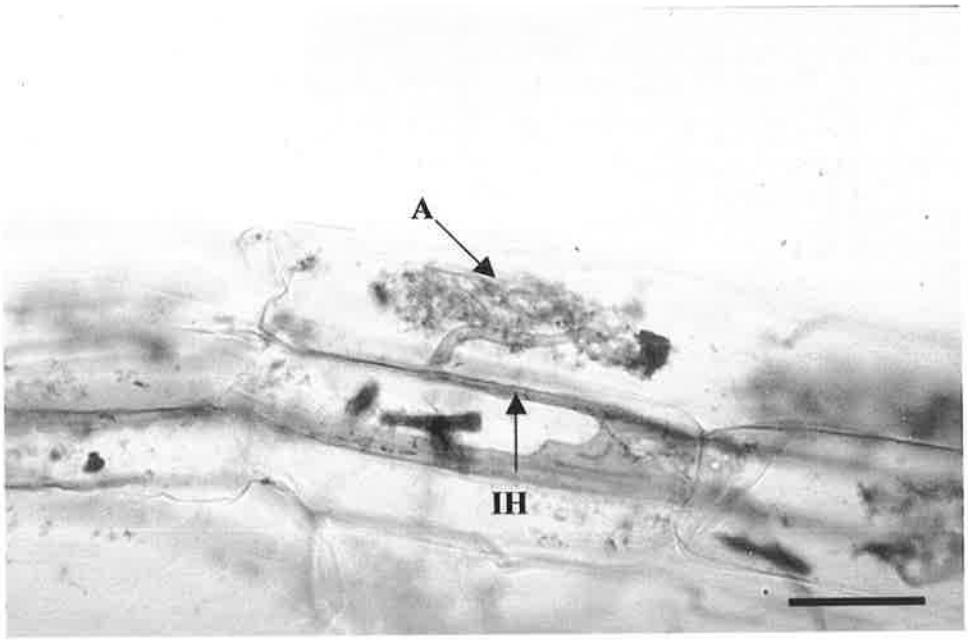


Figure 8.3 Typical arbuscule formed by *G. intraradices* when colonising *Al. porrum*. Note A = arbuscule, IH = intercellular hyphae. Bar = 160 μm.

Differences were apparent within the morphological groups. For example, *G. coronatum, Gi. margarita* and *S. calospora* (all *Paris*-type AM) formed similar numbers of HC but different numbers of AC. This difference can be seen by calculating the ratios of HC to AC, which were 1.0^{a}, 1.4^{b} and 1.8^{c} for *G. coronatum, Gi. margarita* and *S. calospora* respectively (means followed by the same letter are not significantly different at the $p<0.05$ level).

8.4 Discussion

The results presented here demonstrate that the morphology of AM formed by *Ly. esculentum* depends greatly on the species of fungus colonising the roots. This is shown both quantitatively (Figure 8.1 a to f) and qualitatively (Figure 8.2 a to f) in *Ly. esculentum* plants synchronously colonised by the different fungi. The finding is important, as it has been often believed that the morphology of AM is largely dependent on plant identity (Gallaud, 1905; Smith & Smith, 1997, and references therein). Control by plant identity certainly applies to *Al. porrum*, which has been observed by many researchers with many AM fungi (Brundrett *et al.*, 1985; Ezawa *et al.*, 1995; Dickson *et al.*, 1999a, b). It is hence a "robust" *Arum*-type. Such control by the plant presumably occurs in other "robust" *Arum*- or *Paris*-types. Differences in AM morphology when a single plant species is colonised by different fungi have been observed previously (Abbott, 1982; Söderström *et al.*, 1996), although those results were not as dramatic. For example, the present experiment shows changes between *Arum*- and *Paris*-types depending on the fungal species, rather than differences in relative amounts of hyphal coils and arbuscules (Söderström *et al.*, 1996).

The low level of colonisation by *G. coronatum* (Figure 8.1 a) is not consistent with observations from previous experiments (Gao and Cavagnaro, unpublished results) and may be due to poor inoculum quality or unsuccessful

establishment in nurse-pots. Lower than 'normal' levels of colonisation of the *Al. porrum/G. coronatum* nurse-plants support this suggestion.

As well as abundant hyphal and arbusculate coils, *Gi. margarita* and *G. coronatum* formed a few intercellular hyphae (in less than 8 % root length) (see Figure 8.1 d). However, the numbers of intercellular hyphae were very low and I still consider the colonisation pattern to be of the *Paris*-type. Alternatively they might be called "near-*Paris*-type". The boundaries between *Arum*-type and *Paris*-types with respect to intermediate types of AM are not clear cut and intermediate structures in other plant taxa need further study (see Smith & Smith, 1997).

Although not initially considered to be important in controlling AM morphology (Gallaud, 1905), more recent work (Brundrett & Kendrick, 1988) has suggested that the formation of the two types is related to the presence of continuous longitudinal air-spaces in the root cortex, so that hyphae grow along the intercellular air-spaces in the *Arum*-type, and intracellularly in their absence in the *Paris*-type. It then follows that intermediate morphological types may occur in species with discontinuous intercellular air-spaces (Smith & Smith, 1997). Even if the presence and continuity of air-spaces is often the defining morphological character (in terms of plant identity), we now know that fungal identity is also sometimes important, as seen in *Ly. esculentum*. The effect of fungal identity may depend on hyphal diameter or plasticity, that is, the ability of

a fungus to alter its diameter when challenged by an air-space with a diameter less than that of its own hyphae. This situation may be analogous to the narrowing of AM hyphae when forming an infection peg below an appressorium when penetrating roots (Smith & Read, 1997), changing hyphal diameter of pathogenic fungi when penetrating cell walls (Morin, Derby & Kokko, 1996) or to growth of external hyphae in the bulk soil with different textures (E. Drew, personal communications). There is some suggestion that the hyphal diameters in the fungi forming *Arum*-types are less than those of the *Paris*-types (see Figure 8.2 a to f). Furthermore, *G. tenue*, which has a narrow hyphal diameter of 0.5 μm (Schenck & Perez, 1990), has only been observed to form the *Arum*-type in field collected material of *Triticum aestivum* and *T. subterraneum*, some of which apparently formed *Paris*-type AM with "coarse" endophytes (ie, those with wider diameter hyphae) (Z. Antoniolli and S. Smith, personal communication).

In a situation where a fungus is unable to grow along an intercellular space (whether the space is absent, too narrow or discontinuous), successful colonisation will depend on the ability of the hyphae to grow from cell to cell by penetration of the cell walls. Bonfante & Perotto (1995) concluded that penetration of host roots was due to a combination of mechanical and enzymatic mechanisms. A number of enzymes have been identified as having a role in the penetration of cell walls by AMF. These enzymes include cellulase, pectinase and xyloglucanase (see Garcia-Garrido *et al.*, 2000). Similar mechanisms may apply to penetration of cortical cell walls to form the *Paris*-type. Furthermore, these mechanisms may

also influence the ability of a hypha to force its way along a "tight" intercellular space in *Arum*-type AM. Differences in the production of enzymes by a range of *Glomus* species were found by Garcia-Garrido *et al.* (2000). This adds a further layer of complexity to the role of fungal identity on AM morphology.

Attempts to classify plant taxa into distinct AM morphological types (eg Smith & Smith, 1997) are greatly complicated by these results. This is particularly the case with plants collected from the field where different (unknown) AM fungi may dominate colonisation, or where roots may be colonised by more than one AM fungus. It is also possible that reports of plant families having both *Arum*- or *Paris*-type AM, or intermediate types (eg Smith & Smith, 1997) can be accounted for by colonisation by different AM fungi. Plant species with roots containing ever-present, large and continuous air-spaces or limited air-spaces, may always form one type or the other. For example, *Al. porrum* formed the *Arum*-type with the same fungi that formed the *Paris*-type in *Ly. esculentum*. The robustness of presence of air-spaces as a character in plant taxonomy then becomes important, assuming that air-spaces are indeed the definitive character of AM morphology. However, a plant would need to be grown in association with a wide range of fungal species and isolates to be sure that only one morphological type is formed.

Growth of hyphae along air-spaces may be influenced by the extent of existing colonisation. That is, air-spaces may already be occupied by hyphae of

another AMF. For example, up to three hyphae have been observed in the same intercellular space of *Al. porrum* (Smith & Dickson, 1991). Wilson (1984) found differences in the "aggressiveness" of different species of AMF in the roots of *T. subterraneum*. Therefore, competition between hyphae within air-spaces also becomes important. It would be interesting to know the colonisation patterns when *Ly. esculentum* is colonised by mixtures of the AMF used in this experiment.

Within each of the morphological groups variation was noted. For example, *G. coronatum, Gi. margarita* and *S. calospora* (*Paris*-types in *Ly. esculentum*) produced different ratios of HC and AC. If these structures have different roles, these ratios may reflect differences in functionality. There may also be differences in the molecular activity of the interfaces of the structures; however, there have been no molecular studies of *Paris*-type AM (Barker *et al.*, 1998; Harrison, 1999).

The possible role of environmental factors in influencing AM morphology may be important but have not been considered here. Although environmental conditions such as soil P, temperature and light can influence the level of colonisation and relative abundance of structures, at present there is no evidence demonstrating that they change the morphological type of AM. However, flooding of soils can influence the diameter of air-spaces in roots. Therefore, the

potential contribution of such factors in influencing AM morphology needs to be considered in future experiments.

Figure 8.4 is a simple model of the possible pathways a hypha, of large or small diameter, may take where air-spaces are large, absent or of intermediate size. This model assumes that air-space and hyphal diameter are the key determinants of AM morphology. The end result is either an *Arum*-type, *Paris*-type or intermediate type of AM. In the field where a mixed community of AMF exists, potentially with a wide range of hyphal diameters, we may get intermediate types if the plant does not form a "robust" morphological type. It is interesting to note that the original descriptions of the *Arum*- and *Paris*-types come from field collected material where a mixed community of AMF almost certainly existed. Intermediate types may also be formed where the air-spaces are either discontinuous, their diameters change significantly, or the hyphal diameters also change.

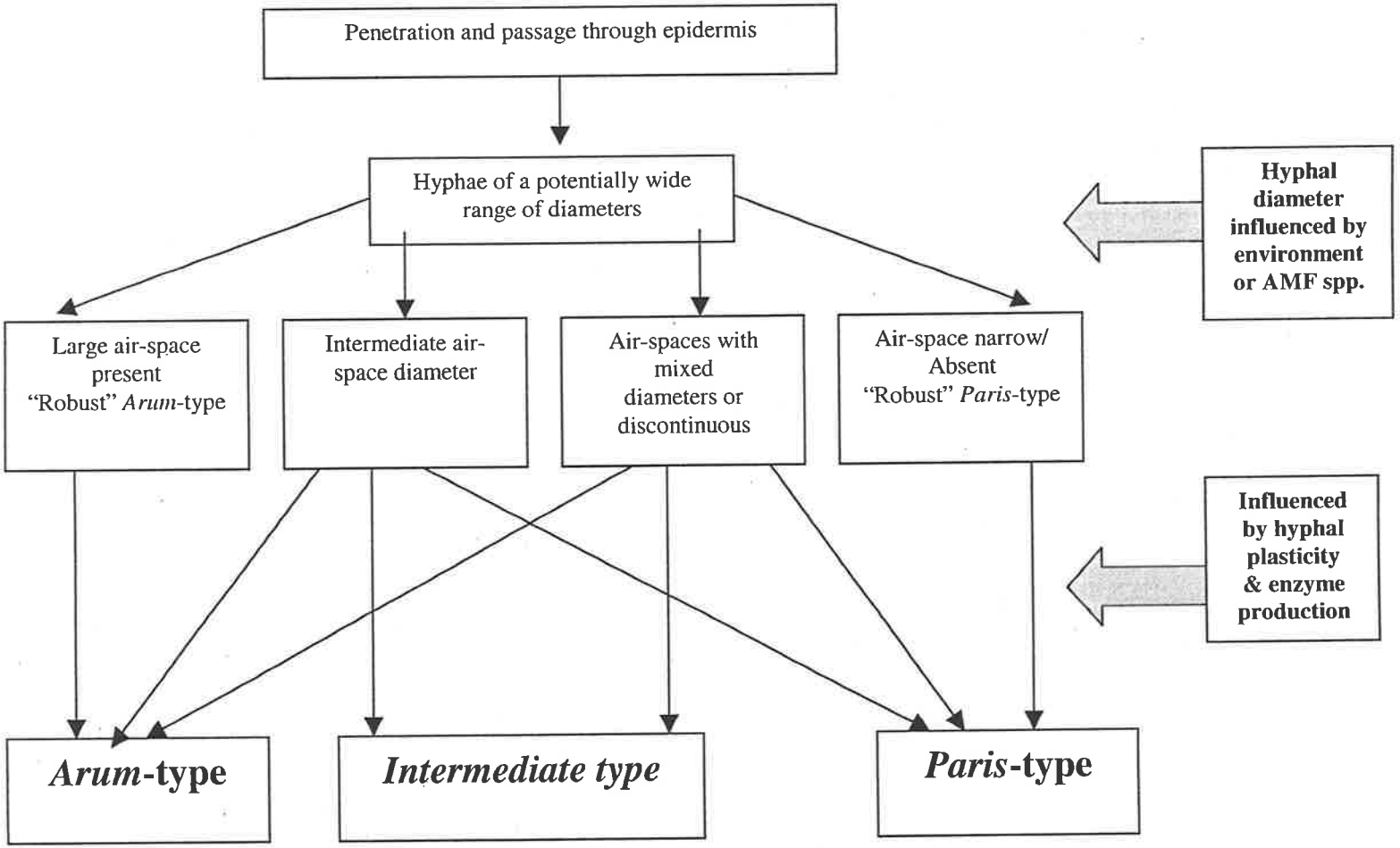


Figure 8.4 Simple model showing passage of hyphae with different diameters within the cortical air-spaces with different diameters, and the resultant AM morphological type.

In order to test this simple model (Figure 8.4) it would be necessary to measure the diameter of both the air-spaces and the hyphae. Measurement of the air-spaces would be possible by infiltrating the air-spaces with a fluorescent dye (such as Sulphorhodamine G) and measuring the diameter and length (continuity) of the spaces using LSCM. Measurement of hyphal diameters is complicated by the fact that the hyphae may alter their diameter when challenged by a narrow air-space. That is, the location at which hyphal diameter is measured may bias the results. Therefore, measurements should be taken immediately outside and inside the root, in intercellular spaces and within cortical cells.

8.5 Conclusions

The morphology of AM formed by *Ly. esculentum* is not solely under the control of the plant genome, but the fungal genome also plays a major defining role. If the presence or absence of continuous longitudinal air-spaces in the roots of plants defines the morphology of AM, the fungi colonising the root will also influence morphology through their hyphal diameter, plasticity, enzymatic and mechanical characteristics. The extent to which these characters differ will clearly depend on the plant and fungal species. Similar diversity in structures may well occur in other AM plants and most probably in plant families that are already known to include *Arum*- and *Paris*-type AM and intermediates. For example, the

Solonaceae have reports of both *Arum*- and *Paris*-type AM (see Table 1.2 in Chapter 1, and McArthur & Knowles, 1992).

In this experiment conventional "root-squashes" for scoring details of colonisation and hence for photography were used. In further experiments root sections and LSCM should be used in order to better visualise the characteristics of the morphological types, especially with regard to differences between the outer and inner cortex of the roots.

The results presented in this chapter have two main implications for AM research. Firstly, investigations where plants are classified as forming either *Arum*-type or *Paris*-type AM, the identity of the fungus/fungi should be reported. Secondly, *Ly. esculentum* (at least this variety) provides a model system in which to study the importance of morphological differences in AM, at not only a structural level, as undertaken here, but also physiological and molecular levels. "Classifications" based on material from the field (for example Smith & Smith, 1997) will be hazardous unless the fungus is known.

Further studies of the role of fungal identity on AM morphology should not only include the cultivar of *Ly. esculentum* used here, but other cultivars, mutants with different root characters (Gao *et al.*, 2001), near relatives, and other members of the Solanaceae.

CHAPTER 9 GENERAL DISCUSSION

9.1 Introduction

The main aim of the work in this thesis was to increase our understanding of *Paris-type* AM. This was done in three stages: firstly, the selection of a plant species forming *Paris-type* AM suitable for use in glasshouse based experiments (Chapters 3 and 4); secondly, the investigation of the development (Chapter 5) and functioning (Chapters 6 and 7) of the selected AM; and thirdly, investigation of the role of fungal identity in AM morphology (Chapter 8). Also it was hoped that an AM symbiosis that lacked arbuscules might be identified, given suggestions in the literature that such AM symbioses exist. In this general discussion I will summarise the key findings of the research, discuss their implications and finally discuss possible future research.

9.2 Discussion

Stage 1 Selection of a suitable *Paris*-type arbuscular mycorrhiza

Most research on *Paris-type* AM comes from field collected material. Although these experiments have provided valuable information, there is also a need for complementary laboratory based experiments. Thus, the first step in this research

was to find a plant/fungus combination which formed a *Paris-type* AM suitable for laboratory based studies.

To this end, a number of plant species were inoculated with two AMF and their morphological development was studied quantitatively. The results (Chapter 3) demonstrate that the development of AM differs not only between plant species but that there are also some quantitative differences between the fungal species tested. Some of the AM investigated did not form the same morphological type reported previously. For example, *L. usitatissimum* and *V. cornuta* both formed intermediate AM morphologies with *Glomus coronatum* and *Scutellospora calospora* although they had been reported to form the *Paris-type* previously (Smith & Smith, 1997). This apparent discrepancy may in part be explained by the results of Chapter 8 (discussed below).

The use of AM in which there is no arbuscular interface would provide a useful system for which the importance of non-arbuscular interfaces in nutrient transfer processes in AM could be investigated. Another aim of the work described in Chapters 3 and 4 was to see if any of the AM tested lacked an arbuscular phase of colonisation. In Chapter 4 an experiment assessing the morphology of AM formed by *Al. porrum* and *S. calospora* at a range of soil P concentrations was conducted. The results of this experiment did not support one previous observation that *Al. porrum* colonised by *S. calospora* does not form arbuscules. The reason for the differences between the two experiments are not

clear; however, the influence of light levels on the production of arbuscules may be important as has been demonstrated previously.

Of all of the plants tested in Chapters 3 and 4, *A. fistulosus* was found to be the most suitable for use in further experiments as it formed a *Paris-type* AM with both *G. coronatum* and *S. calospora*, consistent with observations of field-collected material. The internal morphology of roots colonised by *G. coronatum* was clearer than those colonised by *S. calospora*. Thus, the AM formed by *A. fistulosus* and *G. coronatum* was selected for use in the majority of experiments presented in this thesis (Chapters 5, 6 and 7). Interestingly these two AMF also formed the *Paris*-type in *Ly. esculentum* (Chapter 8).

Stage 2 Morphological development & functioning of *Paris*-type arbuscular mycorrhizas

The time-course of development of a number of *Arum*-type AM have been well described previously. Field-based studies have led to speculation that the time-course of development of *Paris*-type AM may be slower than that of *Arum*-type AM. Time-course studies conducted thus far have used the magnified intersects technique (MIT) which provides a count of intersection containing different AM structures. However, the interdependence of the different structures, that is, their relationships to each other are not taken into account. In Chapter 5 the MIT was modified to give the interdependence magnified intersects technique (IMIT), a

new method which can be used to quantitatively describe the relationships between different morphological features of AM. The IMIT was used to describe the time-course of development of the *A. fistulosus/G. coronatum* symbiosis (Chapter 5).

The time-course of the *A. fistulosus/G. coronatum* symbiosis was slower than that of the *Arum-types* assessed thus far in similar nurse-pot systems. The faster growth rate of hyphae in the intercellular spaces of *Arum-type* roots may be due to lower resistance compared with the intracellular path in *Paris-type* roots (Brundrett *et al.*, 1985). Due to the tortuous nature of the hyphae forming the coils, the same length of hypha may result in a different length of colonisation unit depending on whether it is in the form of intercellular hyphae or hyphal coils.

It was found that hyphal and arbusculate coils were spatially separated, with the arbusculate coils restricted to the inner cortex of the roots and hyphal coils mainly to the outer cortex. The hyphal coils in the inner cortex, were found to be a developmental step of the arbusculate coils. This spatial separation may be in response to different physiological conditions within the two regions of the cortex, or differences in the function of the two types of coils.

Modelling of the data collected using the IMIT demonstrated that the relationships between the different structures were complex, with both time and space influencing the formation of the two types of coils. The statistical

modelling approach showed that the interactions between external hyphae, hyphal coils and arbusculate coils are strong. All three structures are more likely to occur if the other structures are present. The IMIT is a useful method and has other potential applications. It can be applied in any study where the aim is to determine the interdependence of any two or more structures or signals.

In a survey of the literature conducted by Smith & Smith (1997) it was found that *Arum*-type and *Paris*-type AM frequently occurred in different angiosperm families. However, another member of the Asphodelaceae, *Aloe* (Gallaud, 1905) which has a very different root form from *A. fistulosus*, was an *Arum*-type. This highlights the complexity of the basis of the two classes of AM. Use of the IMIT would allow for further detailed comparisons of plants forming the two morphological types.

Plant cells show a range of modifications due to colonisation by AMF. An important response is modification of the size and position of nuclei. This has only been quantitatively investigated in the *Arum-type*, using TEM and conventional light microscopy, that is, two-dimensional analysis. In Chapter 6 the effect of colonisation on the position and size of cortical cell nuclei was measured in three dimensions using LSCM in the *A. fistulosus/G. coronatum* symbiosis.

The volume of the nucleus of colonised cortical cells was determined using stereology as the nuclei did not have a regular shape. It was demonstrated

that in cortical cells containing arbusculate coils, 28 DAT, the volume of the nuclei increased, that is, they were hypertrophied. This is consistent with studies of *Arum*-type AM. The increase in the volume of the nucleus in cells colonised by arbusculate coils is important because it implies the occurrence of similar interactions (potentially decondensation of chromatin) in cells containing both arbuscules and arbusculate coils.

The position of the cortical cell nucleus changed in cells containing arbusculate coils. Low sample-size prevented the collection of equivalent data on hyphal coils, but it seems likely that nuclear reposition occurs and thus implies similar cellular changes. This contrasts with results obtained for hypodermal entry coils (Balestrini *et al.*, 1992) in which there was no nuclear repositioning. The significance of the movement of the plant nuclei in cells colonised by arbuscules, arbusculate and hyphal coils is not well understood. Movement of the nuclei to the centre of the cell allows for closer contact between the plant nuclei and the fungi and may facilitate communication between the symbionts. However, there is no experimental proof to support this speculation and it deserves further study (Barker *et al.*, 1998).

The most widely studied benefit of forming AM is that of improved nutrition, in particular P nutrition. The effect of AMF on the growth and P nutrition under a range of soil P concentrations has been demonstrated many times

for *Arum-type* AM. To my knowledge, the results presented in Chapter 7 provide the first information for a *Paris-type* AM grown under controlled conditions.

Understanding the nutrition of *A. fistulosus* is important as it is a common weed in southern Australia and often invades sites that are nutritionally unfavorable for plant growth. Since *A. fistulosus* benefited from forming AM in soils with low P, in both early and late stages of growth, colonisation may provide an essential advantage in terms of competition and success. Conversely, in higher P soils, colonisation may only benefit *A. fistulosus* if a further stress is imposed upon the plant. The delay in apparent benefit may be of particular importance given that *A. fistulosus* is a perennial herb. Hyphal coils may provide a site of long-term storage of P within roots and should not be overlooked. However, they may also act as a large sink of C also. Given its wide range of different responses to colonisation, *A. fistulosus* is a useful plant to study the physiology and ecology of AM (particularly *Paris-type* AM).

Stage 3 The role of fungal identity on the morphology of arbuscular mycorrhizas

The results presented in Chapter 8 overturn the view that the morphology of AM in *Ly. esculentum* (this cultivar at least) is solely controlled by the plant. This may help to explain reports of plant species forming different AM morphological types. The results presented here show that both the plant and fungus are

important in defining the morphology of AM formed. For example, if the presence or absence of continuous longitudinal air-spaces in the roots of plants mainly defines the morphology of AM, as has been suggested in the past (Brundrett & Kendrick, 1988, 1990b), the fungi colonising the root will also influence morphology through their hyphal diameter, plasticity, enzymatic and mechanical characteristics. The extent to which these characters differ between fungi will clearly depend on the plant and fungal species. The importance of environmental factors should not be ignored for example, flooding of soils can influence the diameter of air-spaces in roots. Therefore, the potential contribution of such factors in influencing AM morphology needs to be considered in future experiments. These results complicate our understanding of AM morphology, but they provide an exciting opportunity to relate structure to function of AM in a single plant species under defined conditions.

9.4 Future Research

As with most research, in the process of answering the questions posed at the beginning of this thesis, many more questions have been uncovered. Some of the many future issues that can be addressed, are as follows:

- It needs to be established if AM morphology is strongly influenced by fungal identity in a wide range of plant species extending from more cultivars of *Ly. esculentum*, through more species of *Lycopersicon* to other members of the Solanaceae. Further attention would be focussed on families listed in Smith & Smith (1997) as having both *Arum*- and *Paris*-types and intermediate morphologies,
- The importance of air-spaces in defining AM morphology needs to be addressed. This would include measurement of the diameter, length and continuity in a range of plant species, both uncolonised and colonised by a range of AM fungi under a range of environmental conditions and over time,
- Chapters 3, 4 and 8 clearly show that the issue of AM morphology is complicated and that careful consideration of the factors controlling AM morphology need to be addressed,
- The time-course of development of more *Paris*- and *Arum*-type AM, with a range of plant and fungal species and environmental conditions, needs to be undertaken. In light of the results of Chapter 8, *Ly. esculentum* may provide a model system for such experiments,

- Hyphal coils and arbusculate coils formed by *G. coronatum* colonising *A. fistulosus* appeared to be spatially separated. The occurrence of this separation in other *Paris*-type AM and the importance in terms of function needs to be investigated,
- It would be interesting to determine quantitatively whether or not hyphal coils have similar effect on cortical cell nuclei as do arbusculate coils and arbuscules, particularly given that the entry coils in the *Arum*-type *Al. porrum* appear to have no effect,
- Attempting to correlate effects on plant nuclei, in colonised cells, to the metabolic activity of the AM structure may be useful in understanding its importance. This would be technically very challenging,
- The P nutrition of the *A. fistulosus*/*G. coronatum* symbiosis was drastically altered by a small addition of P to the soil. This requires further detailed investigation, particularly focusing on biomass allocation in the mycorrhizal and non-mycorrhizal plants,
- The root dry weight of mycorrhizal *A. fistulosus* plants at higher levels of P was much less than that of uncolonised plants. The implications for acquisition of nutrients not readily taken up by AMF and transferred to the plants would be interesting to investigate,
- The development and functioning of the *A. fistulosus*/*G. coronatum* symbiosis should be assessed in field based studies to determine effects on its growth, competitiveness and fecundity over several seasons,

- Importantly *Ly. esculentum* provides a model system in which we can relate structure to function. This can involve investigation of the physiological and molecular differences and similarities between *Arum*- and *Paris*-type AM,
- The use of DNA-fingerprinting to identify unknown AMF may help sort out issues of control of AM morphology in material collected from the field.

In conclusion, when discussing the *Paris*-type Smith and Read (1997) stated…

"…for those interested in the diversity of symbiotic interactions, the study of their occurrence, distribution and function is sure to be rewarding."

-They were right.

APPENDICIES

Appendix 1 Long Ashton solution

Macronutrients	
Nutrient	Concentration
K_2SO_4	2 mM
Mg $SO_4.7H_2O$	1.5 mM
$CaCl.2H_2O$	3 mM
FeEDTA	0.1 mM
$(NH_4)_2SO_4$	4 mM
$NaNO_3$	8 mM

Micronutrients	
Nutrient	Concentration
H_3BO_3	2.86 mg l^{-1}
$MnCl_2.4H_2O$	1.81 mg l^{-1}
$ZnSO_4.7H_2O$	0.22 mg l^{-1}
$CuSO_4.5H_2O$	0.08 mg l^{-1}
$NaMoO_4.2H_2O$	0.025 mg l^{-1}

Appendix 2 Nitric-Perchloric acid

300 ml Concentrated Nitric acid

49.8 ml 70-78% Perchloric acid

Appendix 3 Colour Reagent (for P determination)

1 L Conc. Nitric acid

1 L 0.25% ammonium vanadate

1 L 5% ammonium molybdate

Add acid to ammonium vanadate, mix thoroughly and add ammonium molybdate, mix and allow to cool.

Appendix 4 SDW and RDW of *L. usitatissimum* inoculated with *G. coronatum* and *S. calospora* and not inoculated and grown in the Kutipo and Mallala soils. Values are means +/- S.E. (n=4).

			NM Kuitpo	***S. cal.***	**NM Mallala**	***G. coro.***
SDW (g)	**Harvest 1**	Mean	0.013	0.010	0.009	0.009
		S.E.	0.001	0.001	0.001	0.001
	Harvest 2	Mean	0.023	0.030	0.030	0.051
		S.E.	0.002	0.003	0.008	0.008
	Harvest 3	Mean	0.026	0.146	0.030	0.073
		S.E.	0.002	0.028	0.002	0.004
RDW (g)	**Harvest 1**	Mean	0.011	0.009	0.008	0.007
		S.E.	0.002	0.001	0.001	0.001
	Harvest 2	Mean	0.029	0.023	0.030	0.012
		S.E.	0.003	0.001	0.002	0.001
	Harvest 3	Mean	0.055	0.447	0.105	0.150
		S.E.	0.003	0.020	0.008	0.015

Appendix 5 SDW and RDW of *V. cornuta* inoculated with *G. coronatum* and *S. calospora* and not inoculated and grown in the Kutipo and Mallala soils. Values are means +/- S.E. (n=4).

			NM Kuitpo	***S. cal.***	**NM Mallala**	***G. coro.***
SDW (g)	**Harvest 1**	Mean	0.0023	0.0022	0.0017	0.0015
		S.E.	<0.001	0.001	0.001	0.001
	Harvest 2	Mean	0.0055	0.0079	0.0042	0.0203
		S.E.	0.001	0.001	0.001	0.001
	Harvest 3	Mean	0.0085	0.1212	0.0079	0.0243
		S.E.	0.001	0.009	0.001	0.001
RDW (g)	**Harvest 1**	Mean	Insufficient material for accurate RDW determination.			
		S.E.				
	Harvest 2	Mean	0.0034	0.0056	0.0032	0.0037
		S.E.	0.001	0.001	0.001	0.001
	Harvest 3	Mean	0.0071	0.00768	0.0205	0.0118
		S.E.	0.001	0.001	0.005	0.001

Appendix 6 SDW and RDW of *P. crispum* inoculated with *G. coronatum* and *S. calospora* and not inoculated and grown in the Kutipo and Mallala soils. Values are means +/- S.E. (n=4).

			NM Kuitpo	***S. cal.***	**NM Mallala**	***G. coro.***
SDW (g)	**Harvest 1**	Mean	0.002	0.0013	0.003	0.0015
		S.E.	<0.001	0	<0.001	<0.001
	Harvest 2	Mean	0.002	0.003	0.003	0.003
		S.E.	<0.001	0.001	0.001	0.001
RDW (g)	**Harvest 1**	Mean	Insufficient material for accurate RDW determination.			
		S.E.				
	Harvest 2	Mean	0.013	0.0033	0.003	0.008
		S.E.	<0.001	0.003	<0.001	0.001

Appendix 7 SDW and RDW of *A. fistulosus* inoculated with *G. coronatum* and *S. calospora* and not inoculated and grown in the Kutipo and Mallala soils. Values are means +/- S.E. (n=4).

			NM Kuitpo	***S. cal.***	**NM Mallala**	***G. coro.***
SDW (g)	**Harvest 1**	Mean	0.004	0.006	0.007	0.006
		S.E.	0.001	0.001	0.001	0.001
	Harvest 2	Mean	0.0075	0.0095	0.0075	0.0105
		S.E.	0.001	0.001	0.001	0.001
RDW (g)	**Harvest 1**	Mean	Insufficient material for accurate RDW determination.			
		S.E.				
	Harvest 2	Mean	0.02	0.012	0.01	0.012
		S.E.	0.001	0.001	0.001	0.001

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